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The influence of tannin and tannin with salivary protein on the volatility and the perceived intensity of ethyl hexanoate in a wine-like solution

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A Dissertation

submitted in partial fulfilment

of the requirements for the Degree of

Bachelor of Viticulture and Oenology (Honours)

Lincoln University

by

Yi Yang

Lincoln University

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Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Bachelor of Viticulture and Oenology (Honours).

The influence of tannin and tannin with salivary protein on the volatility and the perceived intensity of ethyl hexanoate in a wine-like solution

by

Yi Yang

The present study investigated the influence of tannin and tannin-mucin interaction on the volatility and the perceived aroma intensity of ethyl hexanoate at 300 μg/L. Data from the instrumental analysis showed that, without mucin, with the increase of tannin concentration, the headspace concentration of ethyl hexanoate was decreased (from 0 to 0.6 g/L of tannins) and then increased (from 0.6 to 16.2 g/L of tannins), in which at 16.2 g/L of tannins, the headspace concentration of ethyl hexanoate measured by HS-SPME-GC-MS was almost the same as found in the control sample that had no tannin. With mucin added to the samples, at higher tannin concentrations, the influence of tannins on the volatility of ethyl hexanoate was disrupted, resulting in the decreased volatility of ethyl hexanoate in the tannin samples with mucin. Sensory experiments were carried out using the method provided by ASTM E679. The results from the sensory experiments illustrated that increasing tannin concentration significantly increased the proportion of panelists (n=36, at 5% significant criterion) correctly choosing the tannin sample as the odd sample. The addition of mucin did not significantly change the sensory responses from the panelists. The group best estimated threshold (BET) values obtained before and after the addition of mucin did not show a significant difference (P>0.05). Therefore, it seemed that the perception changes detected by the panelists were not the result by the changes in the volatility of ethyl hexanoate.

Keywords: Wine aroma, ethyl hexanoate, wine matrix, tannins, retronasal aroma, sensory evaluation, salivary protein, aroma release, HS-SPME-GC-MS, tannin-mucin interaction.

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It has been a journey and I am truly grateful to make it all the way to the end. I still have a lot growing and learning and work to do, and this dissertation is a beautiful sign to continue on that journey.

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Chapter 1

Introduction

1.1 Research Background

It has been shown that wine aroma is one of the most influential factors in consumer preference and the perception of fruity aroma is often assessed as the main sensory criteria positively linked with consumers' liking (Williamson et al 2012). Several recent studies have highlighted the significant contribution of esters to the aromas of both red and white wines (Escudero et al 2007, Jones et al 2008, Pineau et al 2009, Sumby et al 2010, Antalick et al 2014, Ferreira et al 2016). Sumby et al (2010) demonstrated that esters are key aromatic contributors for white wine aromas as they are one of the major compounds of white wine volatile extracts. At low concentrations (typically less than 100mg/L), esters can be associated with fruity characters in wine, whereas at higher concentrations it can result in a solvent like aroma (Sumby et al 2010). It has been reported that even with white wines such as Sauvignon Blanc, which is known to have a few impact compounds (e.g. thiols such as 3-mercaptohexanol) that have dominant influence on the aroma profile, esters still play significant roles in the aroma profile (Benkwitz et al 2012). Baumes et al (1986) identified esters with low detection thresholds that included ethyl acetate (pineapple aroma), ethyl butanoate (apple aroma), ethyl hexanoate (apple skin aroma), ethyl octanoate (fruity aroma), isoamyl acetate (banana aroma or fruity aroma), and 2-phenethyl acetate (tobacco aroma or rose and honey aroma). In addition, esters with fruity characters, such as ethyl propanoate, ethyl isobutyrate, ethyl 2- and ethyl 4-methylpentanoate, ethyl butyrate, ethyl 2- and ethyl 3-methylbutyrate, and ethyl cyclohexanoate, have been identified in red wines (Escudero et al 2007, Pineau et al 2009). Pineau et al (2009) reported that the red berry aroma fractions in their experiment were associated with ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl 3-hydroxybutanoate, while the blackberry aroma fractions were associated with ethyl propanoate, ethyl 2-methylpropanoate and ethyl 2-methylbutanoate. It is suggested that branched esters are associated with blackberry aromas, while straight chain esters are associated with red berry aromas (Pineau et al 2009, Ferreira et al 2016).

During wine tasting, the overall perception of wine aroma results from two main pathways, i.e., the orthonasal and retronasal. Orthonasal olfaction is associated with "the sniff" and describes

the entry of aroma compounds from outside environment through the nostrils to the nasal cavity followed by interaction with odor receptor neurons in the olfactory bulb (Dietrich 2009). Retronasal olfaction is associated with flavor perception and describes the mechanism in which aroma compounds in the oral cavity are released and transported to the back of the nasopharynx (i.e. the soft palate at the back of the nose and connects the nose to the mouth, which allows a person to breath through the nose) and then to the odor receptor neurons (Dietrich 2009). **Figure 1.1** demonstrates these two pathways and aroma compounds in wine are perceived in the olfactory bulbs that house the odor receptor neurons (Dietrich 2009, Maltman 2013). It is suggested that the retronasal pathway occurs when people swallowing the food or beverages, which opens the nasopharynx allowing the transfer of aroma compounds to the nasal cavity. Studies have also shown that the highest amount of aroma transfer via retronasal olfaction happens during the first expiration after swallowing (Buettner and Schieberle 2000, Linforth and Taylor 2000).

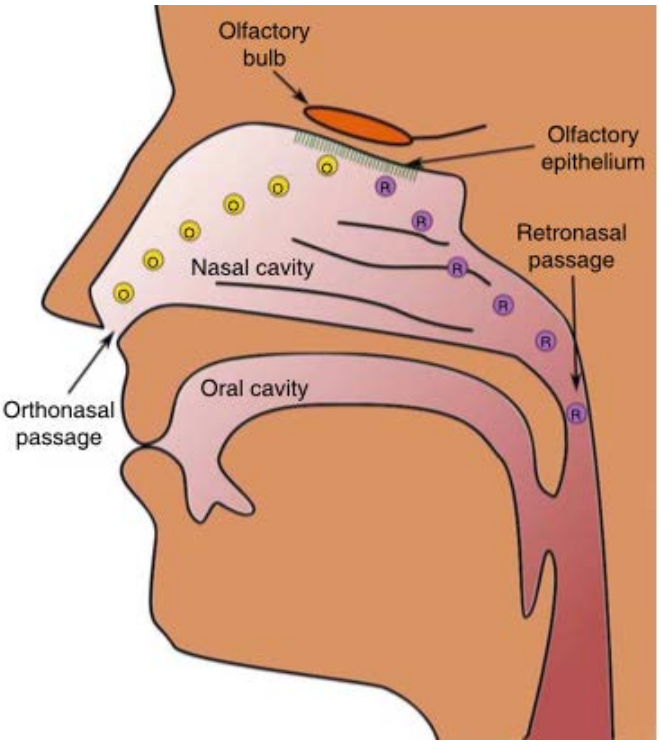

Figure 1.1 Schematic of the human olfactory system by Dietrich 2009

The olfactory system is designed to detect volatile components (Dietrich 2009). The sense of smell and detection of aroma in wine is dependent on the aroma's volatility and affinity for the matrix from which it comes from (Villamor and Ross 2013, Lorrain et al 2013, Lytra et al 2016). In this context, wine has been described as a sensory buffer (Ferreira et al 2010). It contains ethanol and a great number of nonvolatile compounds (Villamor and Ross 2013). As with any

other beverage, interactions between the volatile and the nonvolatile compounds may occur. These interactions may vary depending on the physicochemical properties of the aroma compounds (e.g. molecular size, functional group, degree of hydrophobicity, etc.). For example, binding reactions between volatile and non-volatile compounds may occur via covalent bonds, hydrophobic bonds or hydrogen bonds, or via the formation of inclusion complexes (Mitropoulou et al 2011, Saenz-Navajas et al 2012). These together may regulate aroma compound partitioning between the liquid and gas phases at equilibrium and consequently alter the concentration of free volatile compounds in the headspace responsible for the perception of aroma (Rodriguez-Bencomo et al 2011, Mitropoulou et al 2011, Saenz-Navajas et al 2012, Villamor and Ross 2013, Lorrain et al 2013, Munoz-Gonzalez et al 2015). Several recent studies have evaluated the wine matrix composition of different types of wines (Rodriguez-Bencomo et al 2011, Munoz-Gonzalez et al 2013, Munoz-Gonzalez et al 2015). In these studies, red wines have been found to have significantly higher concentrations of polyphenols and polysaccharides than white wines and sparkling wines (Rodriguez-Bencomo et al 2011, Munoz-Gonzalez et al 2013, Munoz-Gonzalez et al 2015). These variations in the nonvolatile matrix composition between wines might explain why red wines are often found to lack impact aromas compared to other type of wines (Ferreira et al 2010, Antalick et al 2014, Munoz-Gonzalez et al 2015). It has been suggested that the strong retention effects on aroma compounds found in red wines could result from the high content of polyphenols in red wines (Saenz-Navajas et al 2010, Villamor and Ross 2013, Rodriguez-Bencomo et al 2011). A study by Munoz-Gonzales (2013) reported that young and aged red wines showed 10 times more total polyphenols (2010 and 1860 mg/L respectively) compared to white and sparkling wines (211 and 173 mg/L respectively).

Studies have also shown that all aroma compounds do not interact with polyphenol compounds in the same way (Dufour and Bayonove 1999, Jung et al 2000, Aronson and Ebeler 2004, Lund et al 2009, Mitropoulou et al 2011, Goldner et al 2011, Rodriguez-Bencomo et al 2011). Depending on the compound, the reactions may involve interaction or binding with other volatile and nonvolatile compounds, which may affect aroma release and thus the perception of aromas (Goldner et al 2011, Rodriguez-Bencomo et al 2011, Villamor and Ross 2013). There could be a relationship between the length of the chain and hydrophobicity of an aroma molecule and the strength of its interactions with polyphenol compounds (Jung et al 2000, Jung et al 2002). Lorrain et al (2013) found that phenolic compounds have variable impacts on the volatility of aroma compounds, such that catechin had a higher affinity for the esters than gallic acid. In addition, they found that the polarity of the esters as well as their spatial conformation (e.g., branched vs. linear) also had an influence to the interaction strength, i.e., the volatility of the most apolar ester, ethyl octanoate, appeared to be affected by catechin addition the most. In

another study, Jung et al (2000) demonstrated that compared to naringin, gallic acid showed a stronger binding affinity for aromatic compounds such as vanillin, 2-methylpyrazine and ethyl benzoate. In terms of the binding capacity of aroma compounds with the phenolic compounds, 2-methylpyrazine and vanillin showed greater interactions with gallic acid and naringin than ethyl benzoate. Aronson and Ebeler (2004) reported that polyphenols (gallic acid and naringin) induced a greater reduction in the abundance of long-chain esters in the headspace measured using HS-SPME/GC-MS methods. The authors demonstrated that when polyphenols were combined with ethyl hexanoate and ethyl octanoate and ethyl decanoate, respectively, the reduction of ethyl decanoate, with the longest chain, in the headspace was always the highest.

From above it is evident that chemical structures of the aroma and phenolic compounds could play important roles influencing the strength of the interaction. In most of these studies investigating the influence of polyphenols on the volatility of aromatic compounds, their effects on the sensory perception had also been examined. Yet, the examination of sensory perception regarding the interaction between aromatic compounds and polyphenols was often achieved via the orthonasal pathway, whereas the sensory perception via the retronasal pathway has not been studied as much. In the retronasal pathway, saliva has often been thought to play an important role affecting the aroma release from foods, for instance, by the dilution during salivation, and/or the change of pH, and/or the effect of salivary constituents, e.g., mucins. (Harrison 1998, Buettner and Schieberle 2000, Friel and Taylor 2001, Buettner 2002, Linforth et al 2002, Genovese et al 2009, Rinaldi et al 2012). Human saliva is a complex dilute aqueous solution that contains numerous inorganic salts (e.g., sodium, calcium, potassium and bicarbonate) and organic components (e.g., enzymes such as α–amylase and proteases, proline rich proteins, etc.) and proteins (e.g., mucins) (Buettner 2002). Early studies have examined the role of different components (e.g., mucins) or factors (e.g., dilution) of saliva on volatile partitioning from very simple solutions, e.g., water, ethanol as well as oil (Van Ruth et al 2001, Friel and Taylor 2001, Buettner 2002, Linforth et al 2002, Genovese et al 2014). Generally speaking, as examined individually, the components of saliva (e.g., proteins and enzymes) as well as the dilution effect had all shown some impact on aroma release (van Ruth et al 2001, Friel and Taylor 2001, Buettner 2002, Linforth et al 2002). Ployon et al (2017) have reviewed the effects of saliva on aroma release through the interactions between salivary components and aroma molecules, e.g., binding of aroma compounds to salivary proteins and enzymatic conversion of odorant or non-odorant compounds into new odorant compounds. It has been reported that saliva components such as mucins can directly interact with volatile compounds (Friel and Taylor 2001, Pages-Helary et al 2014). Moreover, it has been demonstrated that the retention of ketones and esters by mucin increases, as a result of the hydrophobic interactions

(Pages-Helary et al 2014). Another direct effect of saliva on the release of aroma compounds is through dilution or modification of the matrix or food properties, e.g., the change of matrix pH (Ployon et al 2017). A few studies examined the role of saliva in aroma release during the retronasal pathway of wine consumption using complex wine matrices, e.g., white or red wine extracts (Genovese et al 2009, Munoz-Gonzalez et al 2013, Munoz-Gonzalez et al 2014, Piombino et al 2014). In general, the results from these studies indicate that the influence of saliva in aroma release in red wine matrix is different than in white wine matrix. In these studies, when added with saliva, aroma release in red wine was notable higher than in white wine. Authors of these studies have all suggested that the differences on aroma release between white wine and red wine matrices could be because red wine contains higher amount of total polyphenols. However, there are many other components (e.g., pH, polysaccharides, etc.) in wine and their concentrations are different between red wine and white wine. Mitropoulou et al (2011) demonstrated that the addition of saliva into model wine solution with higher tannin concentration enhanced concentrations of esters (i.e., isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl deacanoate, diethyl succinate, ethyl dodecanoate) in the headspace. However, in this study, the model wine solution to saliva ratio was 3:2, and the ratio used in Genovese et al (2009) and Munoz-Gonzalez et al (2014) was 5:1. van Ruth et al (2001) demonstrated that model solution to saliva ratio could also have a great influence on the aroma release from the solution. For example, the two different model solutions to saliva ratios (3:2 and 5:1) could result in different dilution effects and pH changes, etc., therefore might bring in an array of variations.

1.2 Research Objectives

The overall goal of the present study was to examine influence of tannin and tannin-salivary protein interaction on the aroma release and the perception (i.e., aroma intensity) of ethyl hexanoate in a wine like solution. Ethyl hexanoate was selected on the basis of its structure (i.e., linear) and involvement in wine as well as its odour descriptors (i.e., fruity, strawberry aromas).

***Specific Experimental Objectives were*:**

1. Using sensory panelists to assess the effect of tannin on the perceived aroma intensity of ethyl hexanoate in the wine-like solution examined via orthonasal olfaction.

2. Using sensory panelists to examine the influence of salivary protein mucin on the interaction between tannin and ethyl hexanoate in the wine-like solution examined via orthonasal olfaction.

3. Using instrumental analysis to determine the influence of tannin and tannin-salivary protein interaction on the volatility of ethyl hexanoate in the wine-like solution.

1.3 Human Ethic Approval

All experimentation involving human subjects was conducted in accordance with Lincoln University Human Ethics Committee case approval: LUHEC 2017-21.

1.4 Dissertation Structure

Including this introduction, this dissertation contains five chapters. Chapter 2 reviews the literature related to wine nonvolatile matrix, the influence of tannin on aroma release, major salivary components, the interaction between salivary proteins and volatile compounds, and the interaction between salivary proteins and tannin. Chapter 3 outlines the main methodologies used through out this project. Chapter 4 is the experimental section that contains the results, discussion, and conclusion of the project. Chapter 5 provides an overall summary including comments on future work. Appendix A demonstrates the design for the sensory experiment with randomized and balanced presentation orders. Appendix B provides the scripts used for the warm-up and familiarization session before the sensory experiment. Appendix C provides the sensory results (i.e., individual and group BETs) and the use of statistical analysis in RStudio and is followed by a complete list of references.

Chapter 2

Literature Review

2.1 The Influence of Wine Matrix Components on the Volatility of Volatile Compounds and the Aroma Perception

2.1.1 The Constitution of the Wine Nonvolatile Matrix

Wine has been described as a sensory buffer (Ferreira et al 2010) that contains ethanol and a great number of volatile and nonvolatile compounds (Villamor and Ross 2013). In the assessment of grape juice and wine compositions, it has been shown that the nonvolatile compounds in the matrix are predominantly sugars (i.e. glucose and fructose), polysaccharides, organic acids, amino acids, proteins and phenolic compounds, which come from the skin, seeds and pulp of the grapes, the cell wall of the fermentation yeast, and oak barrels (Nurgel et al 2002, Robinson et al 2009, Rosso et al 2009, Mitropoulou et al 2011, Lorrain et al 2013, Munoz-Gonzalez et al 2015). The quantity and composition of wine nonvolatile compounds can be deliberately modified throughout the fermentation and aging process by choice of winemaking practice, such as juice clarification, maceration, acid adjustment, nutrient addition, protein and phenolic compound additions, barrel aging, and wine clarification and filtration, etc. (Villamor and Ross 2013).

Several recent studies have evaluated the wine matrix composition of different types of wines (Rodriguez-Bencomo et al 2011, Munoz-Gonzalez et al 2013, Munoz-Gonzalez et al 2015). In these studies, sweet wines generally showed highest nonvolatile composition, comparing to white wines, aged and young red wines, and sparkling wines, due to its high residual sugar content. On the other hand, white wines and sparkling wines showed lower nonvolatile composting (i.e. polyphenols, polysaccharides, residual sugars, and nitrogen compounds) when compared to aged and young red wines that had significantly higher levels of polyphenols and polysaccharides (Rodriguez-Bencomo et al 2011, Munoz-Gonzalez et al 2013, Munoz-Gonzalez et al 2015). This nonvolatile matrix effect may lead to various consequences on the perception of wine fruity aromas, which have been reported in a number of studies (Jones et al 2008, Pineau et al 2009, Robinson et al 2009, Saean-Navajas et al 2010, Rodriguez-Bencomo et al

2011, Lytra et al 2013, Munoz-Gonzalez et al 2015, Cameleyre et al 2015). For instance, Saenz-Navajas et al (2010) reported that white wine matrices enhanced the perception of fruity aromas, in which the headspaces above white wine matrices were richer in fruity esters and volatile fatty acids, whereas the influence of red matrices was a lot more complex highly depending on the type of matrix, but in general strongly retained 3-mercaptohexyl acetate that had a significant role in the perception of fruitiness in the white wines. In addition, the authors reported that red wine matrices made white wine aromatic reconstitutions to resemble red wine aromas (i.e. with strong dry fruit, vegetal, and animal notes) and vice versa, highlighting the power of nonvolatile matrix in affecting the perception of wine aroma.

2.1.2 The Influence of tannin on the release of wine volatile compounds and the aroma perception

As shown earlier, the strong retention effects on aroma compounds found in red wines could result from the high content of polyphenols in red wines (Saenz-Navajas et al 2010, Villamor and Ross 2013, Rodriguez-Bencomo et al 2011). Rodriguez-Bencomo et al (2011) reported that aroma compounds' interactions with aged red wine matrix were significantly stronger than with sweet wine. The authors suggested that the presence of higher polyphenols in red wines might be responsible for this effect. A study by Munoz-Gonzales (2013) reported that young and aged red wines showed 10 times more of the total polyphenols (2010 and 1860 mg/L respectively) compared to white and sparkling wines (211 and 173 mg/L respectively). The composition of polyphenols in wines is dependent on the grape and winemaking practices with reactions begin as soon as the grapes are crushed and pressed and continuing through winemaking and aging (Villamor and Ross 2013). Studies have shown that aroma compounds do not interact with polyphenol compounds in the same way (Dufour and Bayonove 1999, Jung et al 2000, Aronson and Ebeler 2004, Lund et al 2009, Mitropoulou et al 2011, Goldner et al 2011, Rodriguez-Bencomo et al 2011). For example, Dufour and Bayonove (1999) found that tannin addition (from 0 to 5 g/L) significantly increased the volatility of limonene and slightly increased the volatility of benzaldehyde but had no effect on isoamyl acetate and ethyl hexanoate. In another study, Lund et al (2009) reported that different polyphenols (i.e. catechin, caffeic acid, quercetin) had different effects when combined with a specific aroma compound (e.g. 3MH, 3MHA or ethyl decanoate) and either suppressed, or accentuated or had very little effect on the perception of the aroma. For instance, the addition of catechin (10 mg/L) and quercetin (10 mg/L) respectively to the diluted model wine of Sauvignon Blanc (6.25% ethanol) both increased the sensory threshold of 3MH (determined in the Sauvignon Blanc wine), in which quercetin

increased the sensory threshold of 3MH by 100%, whereas adding caffeic acid (10 mg/L) had reduced the sensory threshold of 3MH. Comparing to 3MHA and ethyl decanoate, the concentration of 3MHA in the headspace and its aroma perception were the least affected by the added polyphenols, possibly due to the presence of the acetate group in 3MHA.

It is suggested that chemical structures of the aroma and phenolic compounds could play important roles influencing the strength of the interaction. For example, there could be a relationship between the length of the chain and hydrophobicity of an aroma molecule and the strength of its interactions with polyphenol compounds (Jung et al 2000, Jung et al 2002). Indeed, Aronson and Ebeler (2004) reported that polyphenols (gallic acid and naringin) induced a greater reduction in the abundance of long-chain esters in the headspace measured using HS-SPME-GC-MS methods. The authors demonstrated that when the two polyphenols were respectively combined with ethyl hexanoate and ethyl octanoate and ethyl decanoate, the reduction of ethyl decanoate, with the longest chain, in the headspace was always the highest. Similar observations were reported in Mitropoulou et al (2011) who also found that seed-derived tannins and skin-derived tannins had different effects on the release of some aroma compounds. It has been demonstrated that grape skin contains the highest amount of tannins, which are different from seed tannins in terms of their degree of polymerization and amount of gallates (Pinelo et al 2006). The average polymerization degree for skin tannins is around 28, while the seed tannins are more in monomeric form rather than polymerized with an average degree of 11(Pinelo et al 2006). In Mitropoulou et al (2011), increasing the concentration of skin-derived tannins (0-5 g/L) significantly decreased the volatility of longer chain ethyl esters (i.e. ethyl octanoate, ethyl decanoate and ethyl dodecanoate) and isobutanol and linalool, but increased the volatility of the hydrophilic compounds such as, isoamyl acetate, 2-methyl-1-butanol, diethyl succinate and phenylethyl alcohol. They suggested that the decrease of the volatility of the 3 longer chain ethyl esters and isobutanol and linalool was due to their interactions with tannins through hydrophobic bound and/or hydrogen bound to form colloidal size particles. The significantly lower retention effects on the hydrophilic compounds could be due to these compounds are more soluble in water and can also diffuse more easily through the matrix. This suggests that the result of the interactions between skin-derived tannins and aroma compounds is dependent on the physicochemical properties of the aroma compound. On the other hand, increasing the concentration of seed-derived tannins (0-5 g/L) had almost no influence on the volatility of the studied compounds with only a slight salting-out effect observed on ethyl dodecanoate and octanoic acid. However, the volatility of these two compounds decreased significantly when the concentration of the seed-derived tannin increased to 10 g/L. This suggests that in the wine matrix skin-derived tannins may have a

stronger influence on the release of aroma compounds over seed-derived tannins, which may be due to their higher degree of polymerization.

2.1.3 Summary

It is well known that wine matrix components interact with aroma compounds, which may modulate their volatility and headspace partitioning and consequently the perception of aromas. Interactions between volatile and wine matrix compounds not only depend on the type and the concentration of the matrix compound, but also on the physiochemical properties of the aroma compound, such as hydrophobicity and the chain length of the molecule. As the result of these interactions, a retention effect may be induced, where a decrease in the amount of aroma in the headspace may be observed, or a salting-out effect may be obtained, where an increase in the volatilities of some aroma compounds may be found. For instance, with a given compound, its volatility may be increased or decreased as a result of the increase of tannin concentration. Moreover, the wine matrix contains a great number of compounds that may interact with each other, therefore the release or retention of aroma compounds is also affected in varying degrees by the presence of multiple interactions among wine matrix components. To understand the influence of each matrix component on the aroma release and the aroma perception, each matrix component should be studied individually first.

2.2 Retronasal Aroma: the Influence of Saliva on the Volatility of Aroma Compounds

2.2.1 Major Salivary Components

Saliva plays an important role in sensory perception of food and beverage. During eating/drinking, salivation, chewing and temperature are factors that can modify the sensory properties of the food/beverage (Buettner and Schieberle 2000, van Ruth and Buhr 2004). Salivation has a prominent role in retronasal aroma perception, which has been studied by various authors (Harrison 1998, Buettner and Schieberle 2000, Friel and Taylor 2001, van Ruth et al 2001, van Ruth and Buhr 2004, Carvalho et al 2006). The hydration and/or dilution of foods by saliva can affect the partitioning of aroma compounds between food, saliva and air-phase (van Ruth et al 2001). In addition, the saliva to food ratio has been found to determine the extent of hydration and dilution, which is another important factor for aroma release. On the other hand, salivary components can also have a huge impact on the solubility and availability of volatile compounds, thus affecting the concentration of compounds between the liquid and gaseous phases (Friel and Taylor 2001, van Ruth et al 2001).

Saliva is a complex hypotonic fluid that contains 98% water and numerous inorganic and organic compounds. It contains various electrolytes with sodium, calcium, potassium, chloride, phosphate, and bicarbonate being the principal ions (Friel and Taylor 2001). The concentration of bicarbonate regulates the salivary pH and is highly dependent on the type of salivary gland from which it originates (Friel and Taylor 2001, Piombino et al 2014). Generally speaking, the pH of saliva can range from 6.2 to 7.4 (Friel and Taylor 2001). In food or beverage with low pH, such as wine, saliva's close to neutral pH can shift the relative equilibrium of volatile compounds between liquid and air phase, which may affect the aroma perception (Roberts and Acree 1995, van Ruth et al 2001).

Besides these inorganic salts, saliva also contains a great diversity of organic components, including enzymes (i.e., esterases, amylase, etc.), immunoglobulins, and proteins such as the histatins, statherin, Immunoglobulin A (IgA), glycoprotein mucin and proline-rich proteins (Asquith et al 1987, Humphrey and Williamson 2001, Sarni-Manchado et al 2008, Friel and Taylor 2001). Denny et al (2008) revealed that saliva contains at least 1166 proteins, among which mucins and α-amylase are the most abundant. Most proteins found in saliva are made by the salivary glands, but there are large differences between the glands as to which proteins they

synthesise (Dodds et al 2005, Carpenter 2013). Some proteins are universal to all glands, while others are synthesised only by certain glands (Carpenter 2013). For instance, mucins (i.e., products of Muc5b and Muc7 genes) are common to the submandibular and sublingual glands as well as most minor glands but are not syntheised by the parotid and von Ebner's glands (Carpenter 2013). Carpenter (2013) suggested that because submandibular and sublingual exit into the mouth under the tongue, these two glands may contribute the most to resting saliva. Furthermore, saliva produced by submandibular and sublingual glands are mostly distributed across the mouth by the action of the tongue (Carpenter 2013). Mucins are high-molecular-weight glycoproteins with an elongated structure and can self-aggregate to form very large structures, which are responsible for the viscosity of whole-mouth saliva or submandibular/sublingual saliva (Carpenter 2013). These properties of mucins have given saliva several functions, including lubrication of the oral surfaces during the action of eating and drinking as well as maintenance of a hydrated layer covering epithelium (Bansil and Turner 2006). The proline-rich proteins in saliva (i.e., acidic, basic, and glycosylated PRPs) synthesised mainly by the parotid glands, constitute approximately 70% of the total content of parotid saliva (Carlson 1993, Dodds et al 2005). The parotid gland produces very little amount of saliva at rest but delivers the most during periods of chewing (Carpenter 2013). The enzyme amylase comprises most of the rest of the total protein content of parotid saliva (Dodds et al 2005). Amylase is the single most abundant protein in saliva (Carpenter 2013). It is generally thought to be responsible for the initial starch hydrolysis of starch-containing foods. Moreover, amylase is also very efficient at converting many non-soluble complex polysaccharides into smaller soluble units (Dodds et al 2005, Carpenter 2013).

As a protein-rich solution, saliva's macromolecular influence on volatility of aroma compounds, and consequently to modify aroma perception, has been well documented (Asquith et al 1987, Friel and Taylor 2001, Sarni-Manchado et al 2008, Guichard 2006, Genovese et al 2009, Mitropoulou et al 2011). Different mechanisms including non-covalent or covalent binding, interaction with the food matrix should be considered when considering the role of salivary proteins in aroma release. These are discussed in the next two sections.

2.2.2 The Interaction of Volatile Compounds with Salivary Proteins

Delivery of aroma compounds to the olfactory epithelium in the nose can take place orthonasally via the nostrils during inhalation or retronasally when food is eaten (Linforth et al 2002). In the later, aroma compounds are released during mastication in the mouth and travel into the nasal cavity. Differences between orthonasal and retronasal perception of the same

aroma compound have been reported, indicating an influence of oral processing on aroma perception (Linforth et al 2002). It is well known now that when volatile compounds are released from a food matrix into the saliva, interactions may occur either between the volatile compounds and solutes (i.e., salts or sugar) and/or between volatile compounds and proteins (Friel and Taylor 2001, van Ruth et al 2001). Because mucins and α-amylase are the most abundant proteins in saliva, numerous studies have used artificial saliva containing either one of two proteins or both proteins to investigate the effect of salivary proteins on aroma release. So far, mucal proteins have been identified as the key component in saliva affecting aroma release (van Ruth et al 1995, Friel and Taylor 2001, Ployon et al 2017). Mucins are globally negative charged high-molecular-weight glycoproteins that have binding sites, preferentially occupied by sucrose, available to trap some volatile compounds (Friel and Taylor 2001). By trapping volatile compounds at the binding sites via hydrophobic interactions, mucins can reduce their effective concentration in solution and thus, reduce their concentration in the headspace. A decrease of the volatility of a wide range of volatile compounds in the mucin-containing saliva was reported by van Ruth et al (1995). Moreover, the authors found that more hydrophobic and less volatile compounds were more likely to be affected by the presence of mucal proteins, resulting in further decreased volatility. Similar results were reported by Pages-Helary et al (2014) who observed that the presence of mucin induced an increase in the hydrophobicity of the selected ketones and esters. In another study, van Ruth et al (2001) found that the addition of mucins resulted in a salting-out effect for hydrophilic compounds (i.e., dimethyl sulfide, propanol, diacetyl, 2-butanone and ethyl acetate) and a binding effect for hydrophobic compounds.

Friel and Taylor (2001) studied fourteen aroma compounds and reported that the volatility of most volatile compounds was negatively affected by the presence of mucin and further decreased by adding salivary salts, but was increased after the addition of sugars. It was found in mucins that, attached to the protein chain, the charged oligosaccharide side chains were able to interact with small-molecular-weight solutes (i.e., salts and sugars) by modifying the charge repulsion between each glycoprotein molecule (Harding 1989). Hydrogen ions and cations from salivary salts were found to be able to cause a charging-shielding effect, which could affect the charge of the proteins and thus lead to the modification of mucin such as self-aggregation and change of the viscosity (Ployon et al 2017). Friel and Taylor (2001) argued that this modification could then alter the amount of free space between the mucin molecules and consequently affect the interaction of mucins with volatile compounds. Moreover, the authors suggested that this type of interactions should also differ depending on the structure of volatile compound, as the volatility of cymene, decanal, decanol and heptanal was not affected at all by the addition of salts. In terms of the influence of sugars, the authors argued that the resulted increase of the

volatility suggested a competition between aroma compounds and sugars on the binding sites of mucins. Yet, the volatility of aroma compounds such as cymene, decanal, decanol and heptanal was not affected by the addition of sugars. Therefore, this could suggest an existence of different types of binding sites on mucins, which could be affected differently by the competition between volatile compounds and sugars (Ployon et al 2017).

Amylase is the second most abundant salivary protein that exhibits a well-defined three-dimensional structure (Ployon et al 2017). The role of α-amylase in saliva is generally thought to be involved in the initial hydrolysis of starch-containing foods. It is very efficient at converting maltose to glucose as well as converting many non-soluble complex polysaccharides into smaller soluble units (Carpenter 2013). Regarding its enzymatic functions, it has been discussed in Carpenter (2013) and Ployon et al (2017). Pages-Helary et al (2014) reported that the addition of human α-amylase induced a decrease of the concentration of ketones and esters in the headspace. In addition, they found that retention on volatile compounds was negatively correlated to the hydrophobicity of those compounds, suggesting the binding via hydrophobic interactions between volatile compounds and hydrophobic domains of the protein. They also proposed that the presence of both mucins and α-amylase could affect the total number of binding sites of the proteins available for aroma compounds. However, the authors did not observe a cumulative effect of saliva containing both mucins and α-amylase on the volatility of aroma compounds. In an earlier study, van Ruth et al (1995) reported that adding α-amylase to the salt and mucin containing solutions resulted in insignificant changes in the headspace concentration of volatile compounds. The authors suggested that in low-starch systems, α-amylase has no further effect on volatile release from saliva solution. In a later study, van Ruth and Roozen (2000) demonstrated that aroma release from high-starch foods was affected by α-amylase when α-amylase and food were in contact for an extended period of time in a model mouth system. However, the result found *in vivo* did not reflect this situation (van Ruth and Roozen 2000).

Besides the influence of mucins and/or α-amylase on aroma release, several studies have investigated the influence of whole human saliva on aroma release. In terms of the differences between the influence of human saliva and artificial saliva, the results varied between studies. The study by van Ruth and Roozen (2000) did not observe any significant difference on aldehyde release from rehydrated bell peppers between the presence of whole human saliva and artificial saliva. In contrast, Pages-Helary et al (2014) reported that human saliva resulted in a stronger retention effect on ester release than the use of artificial saliva. Similar to Pages-Helary et al (2014), Genovese et al (2009) reported that more aroma compounds in wines were affected by

human saliva than artificial saliva. The difference observed in these studies could be due to the variability in human saliva composition and/or by the different physiochemical properties of the aroma compounds that were studied (Ployon et al 2017).

2.2.3 Influence of the Interactions Between Salivary Proteins and Tannin on the Volatility of Aroma Compounds

In order to understand the effect of saliva on aroma perception in a certain type of food, it is important to incorporate the food matrix in which aroma compounds are initially present. Many studies have reported the interaction between tannin and proteins (Sarni-Manchado et al 1999, Mitropoulou et al 2011, Canon et al 2013, Munoz-Gonzalez et al 2014). Tannins are able to bind salivary proteins, especially mucins and proline-rich proteins to form soluble and non-soluble complexes (Sarni-Manchado et al 1999, Canon et al 2013). For instance, the highest polymerised tannins mainly precipitate together with the salivary protein, and for low polymerised tannins, tannin-protein interactions lead to the formation of complexes that remain soluble (Sari-Manchado et al 2008). The concentrations of tannin can vary greatly between red wines and white wines. Generally speaking, white wines have a lower tannin concentration and the degrees of polymerisation of tannins in white wines are lower than in red wines. The types of tannin in wine and their influence on aroma release have been discussed earlier in **Section 2.12**.

In the study by Genovese et al (2009), the authors noticed a stronger retention on aroma release by saliva in white wine than in red wine. They suggested that this could be due to in red wine tannin-salivary protein interactions inhibited the binding of aroma compounds to mucins. In contrast to Genovese et al (2009), Munoz-Gonzalez et al (2014) reported that, in general, adding saliva decreased the aroma release of various aroma compounds, but red wines were more affected than white wine. The authors argued that in this case the formation of tannin-salivary protein complexes could retain volatile compounds in the hydrophobic cavities and therefore, decrease aroma release into the headspace. Furthermore, they reported that hydrophobic aroma compounds have shown higher retention in red wines with saliva than in white wines. In another study, Mitropoulou et al (2011) reported that adding artificial saliva to the reconstituted red wine decreased the aroma release of the more hydrophilic compounds but enhanced that of hydrophobic compounds. The authors suggested that the tannin-salivary protein complexes could expel hydrophobic compounds from the red wine matrix. Munoz-Gonzalez et al (2014) suggested that the influence of tannin-salivary protein complexes on aroma release is dependent upon the physiochemical properties of the aroma compounds and

perhaps the structure of the complexes as well (i.e., based on the types of tannin and proteins). Both studies by Genovese et al (2009) and Munoz-Gonzalez et al (2014) had also compared the differences between the influence of human saliva and artificial saliva, and found that human saliva had stronger effects than artificial saliva. This could be due to some specific interactions between tannins and other proteins in the real human saliva (such as proline-rich proteins that were not included in the artificial saliva in both studies), which were also interacting with aroma compounds. For instance, proline-rich proteins have demonstrated a high affinity for tannins, resulted by their extended conformation, i.e., with the presence of several proline clusters in their sequence leading to high protein-tannin stoichiometries (Canon et al 2013, Ployon et al 2017). Moreover, it has been suggested that the aggregates of proline-rich proteins could interact with aroma compounds differently to the complexes of tannin-proteins (Canon et al 2013).

2.2.4 Summary

The influence of saliva on the volatility of aroma compounds may be resulted by direct hydrophobic bindings between salivary proteins and the aroma compounds and/or via interactions among salivary proteins, matrix components and the aroma compounds. Regarding the aroma compounds, volatiles that are released between oral gaseous phase, saliva, and matrix are closely dependent on their chemical structure and their affinity for each phase. Aroma compounds show a large diversity of chemical structures and physicochemical properties. Therefore, these can also modulate the influence of salivary proteins. When food and beverages are consumed, the interaction between the food matrix and saliva occurs in the oral cavity. During winetasting, interactions between tannins and salivary proteins can occur, which affects the aroma release of volatile compounds in wine. Tannins may bind to salivary proteins, occupying the binding sites on salivary proteins, which may therefore reduce the retention effect of salivary proteins on aroma compounds. On the other hand, the formation of tannin-protein complexes, due to their interactions, may encapsulate aroma compounds and consequently reduce the volatility of aroma compounds in wines. However, these two possible pathways of tannin-protein interactions and their influence on aroma release during winetasting have not been very well investigated. Moreover, most studies investigated the role of saliva in aroma release did not examine the impact of saliva on aroma perception. Therefore the influence of saliva on the actual wine aroma perception is not very clear, as the amount of volatiles present in the nasal cavity does not necessarily correlate well with sensory perception. Future work will require more detailed investigations combining sensory analysis (i.e., on aroma perception), instrumental analysis (i.e., on the headspace concentrations of volatile compounds)

and saliva biochemical analysis, in order to have a better understanding on the influence of saliva in aroma perception.

Chapter 3

General Methodology

3.1 Overview

This section consists of all the general methods and materials used in the experiment, including the background of the sensory method ASTM E679; the recipes for making the wine-like solutions and other reagents used for the sensory experiment and instrumental analysis; test sample preparations; the preliminary experiments; and the use of instrumental analysis. Additional information regarding the general conditions for sensory evaluation, the procedures used in the sensory experiment, and the statistical method used in data analysis can be found in the next section.

3.2 Method for Sensory Experiment

3.2.1 ASTM International Standard Practice E679 (ASTM E679)

ASTM International (American Society for Testing and Materials International) is a voluntary organization that develops and deliveries consensus standards for a wide range of materials, products, systems, and services (www.astm.org). ASTM E679 was designed as an attempt to achieve method standardisation for measuring odour detection thresholds (Peng et al 2012). It was originally published in 1997, revised in 2004 and recently reapproved in 2011. It has been recognised as a simple and rapid testing method due to its data collection procedure (Lawless and Heymann 2010). It prescribes the use of the method of limits with only an ascending concentration series using the 3-alternative forced-choice (3-AFC) test at each chosen concentration step to find individual and group odour thresholds. All participants receive two control samples and one spiked sample, giving three possible presentation orders, i.e., CCS, CSC, SCC. The use of an ascending series is because it is considered that olfactory receptors are highly susceptible to adaptation (Lawless and Heymann 2010). The concentration steps are separated by a constant factor. The testing procedure of ASTM E679 consists of several concentration steps (generally 6 to 10 steps). For each 3-AFC presentation, the panelist is required to indicate which of the samples is different from the other two. A choice must be made, even if the panelist cannot identify a difference between the samples. The correctness of the judge's

response for each 3-AFC test (i.e., correctly identified the odd sample) constitutes the response dataset.

The instruction given by ASTM E679 for the 3-AFC task is different from the instruction given for the same test by the International Organisation for Standardisation (ISO 13301:2002). This instruction is similar to the instruction given for the triangle test (ISO 4120:2004). For the instruction given by ASTM E679, the panelists are asked to identify the odd sample, whereas the instruction of the conventional 3-AFC test asks the panelist to identify the target sample (e.g., "which sample is the most…?"). In sensory discrimination analysis, the type of instruction is very important, as it determines the panelist's choice of cognitive strategy. During discrimination testing, there are generally three distinct successive stages. First, the stimuli is perceived by the panelist and the sensory characteristics are stored into his/her memory; second, cognitive processes are used by the panelist using the perceived and memorized sensations to try to resolved the specified question or the unspecified question from the instruction; third, an answer is given by the panelist that is based on the combination of the information available and the underlying cognitive process (Rousseau 1999). The theoretical basis for discrimination tests is Thurstonian modeling. It is derived from Thurstone's law of categorical judgment (Thurstone 1927) and further advanced based on the paralleled signal detection theory (SDT) (Green and Swets 1966). This modeling is built on two assumptions: the variability of sensory perception and the use of a decision rule (or sometimes called the cognitive strategy) to generate an answer (O'Mahony and Rousseau 2002, Ennis et al 2014, Rousseau 2015). During sensory evaluation, products are tasted in a sequence and often resampling is allowed, which induces sensory adaptation and generates random noise in the assessor's nervous system. This is because residual stimuli from the previous sample cause variability in the number of active sensory receptors in the peripheral system (O'Mahony and Rousseau 2002). Also, there can be variations both within and between samples due to non-homogeneity in the product (O'Mahony and Rousseau 2002). Therefore, given the first assumption, the intensity of product perception is not constant, but varies slightly according to a frequency distribution usually accepted to be normal (O'Mahony and Rousseau 2002).

The cognitive processes associated with the triangle test (i.e., comparison of distance strategy) and the 3-AFC test (i.e., skimming strategy) have been illustrated in O'Mahony et al (1994) and Rousseau (1999). Following the skimming strategy, a panelist would more likely to pick the sample that elicits the most intensive sensation. Following the comparison of distance strategy, it is suggested that a panelist would use the absolute perceptual distance between the stimuli to make the judgment (Rousseau 1999). In theory, both cognitive strategies could lead to the

wrong judgment (Rousseau 1999, Lawless and Heymann 2010). For example, **Figure 3.1**, **3.2** and **3.3** demonstrate the three scenarios where these strategies can lead to the correct and also the wrong judgment. In these three scenarios, sample A is, for example, the blank control, while B is a sample to be tested (e.g., sample spiked with a substance). In **Figure 3.1**, scenario 1, it can be seen that both comparison of distance and skimming strategy lead to the identification of the test sample (i.e., odd/target sample B) that is the most different/the most intensive sample. In **Figure 3.2**, scenario 2, the comparison of distance strategy leads to a wrong judgment as it misidentifies the blank sample 1 as the most different sample (i.e., the odd one). However, in the same scenario, the skimming strategy leads to a correct response, based on the target sample is perceived as the most intensive sample. On the contrary, in **Figure 3.3**, scenario 3, it can be seen that the skimming strategy leads to the misidentification of the target sample, whereas the comparison of distance strategy leads to the correct judgment, as the odd sample is the most different sample.

Studies have demonstrated that the skimming strategy is a more optimal strategy (Frijters et al 1980, Ennis 1993, Ennis and Jesionka 2011, O'Mahony et al 1994, O'Mahony, 1995). In these studies, comparing the two strategies in the 3-AFC and triangle tests respectively with the same products, the 3-AFC test consistently resulted in a larger proportion of correct responses than the triangle test. Because the sensory instruction prescribed by ATSM E679 is similar to that of the triangle test, therefore, it is very likely that panelists will employ the comparison of distance strategy (Peng et al 2012). To modulate the effects of the instruction given by ASTM E679 on choice of cognitive strategy, it is suggested that panelists can be informed that the odd sample is always the target sample (Peng et al 2012). For instance, in Peng et al (2012), panelists were told that each 3-AFC trial included two odourless blank samples and one odd sample that had either higher or lower intensity compared to the other two. The authors argued that by informing the panelists with this additional information, i.e., the target sample is always the odd sample, thus cognitively choosing the odd sample is equivalent to choosing the target sample. Studies by Ross et al (2014) and Perry and Hayes (2016) also considered this issue and chose to treat each sensory test in ASTM E679 as the triangle test and later process the data (i.e., the correct sensory response) using the table of the critical values (i.e., the number of correct responses needed to reach 5% significance criterion) for the triangle test.

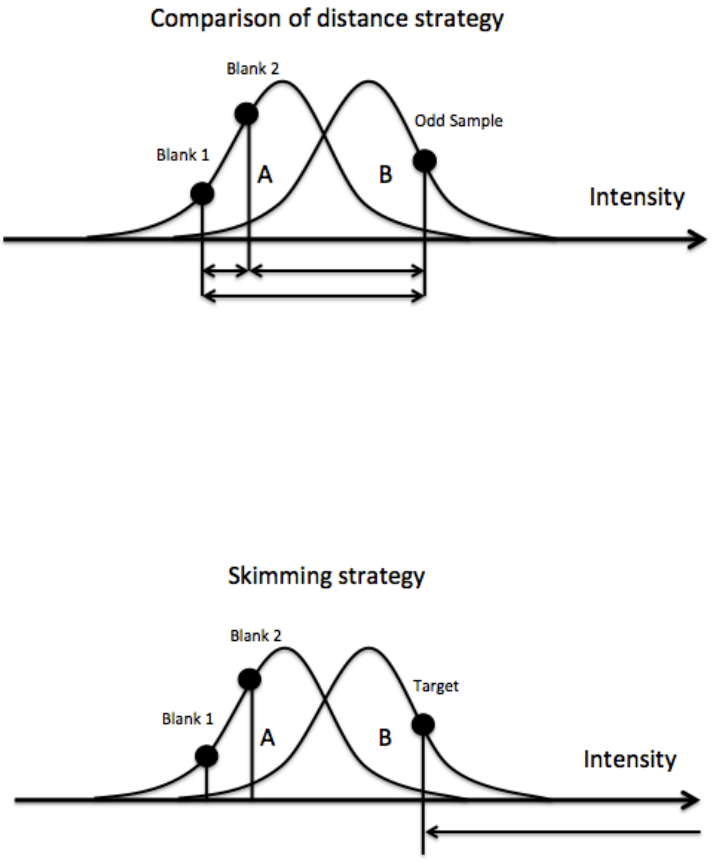

Figure 3.1 Scenario 1: Both comparisons of distance and skimming strategy lead to a correct response.

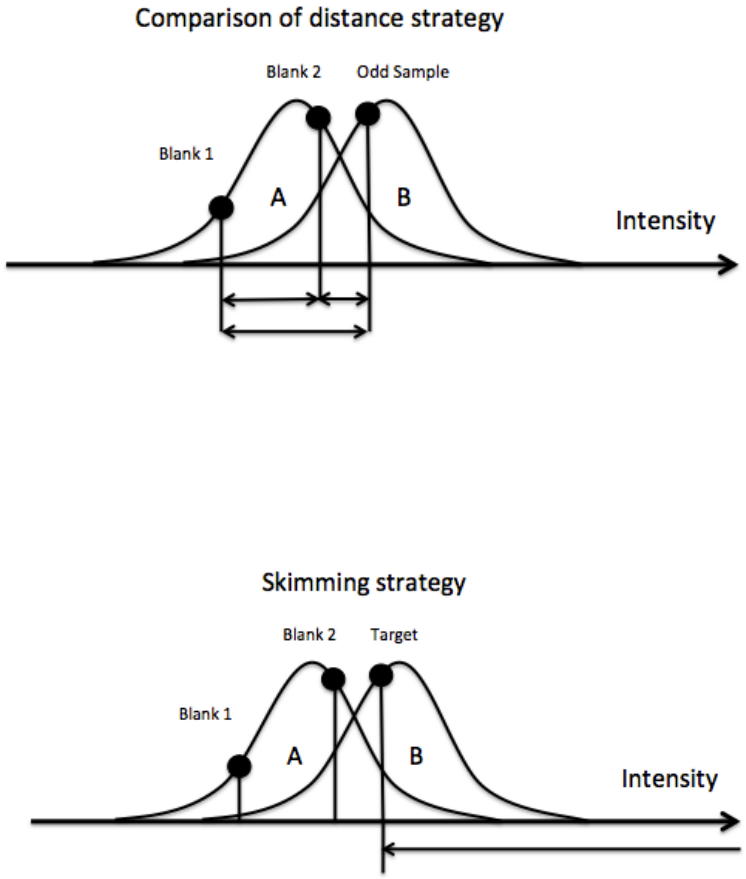

Figure 3.2 Scenario 2. With the same sensory evidence, comparison of distance strategy leads to a misidentification of the odd sample, whereas skimming strategy leads to a correct identification of the target sample.

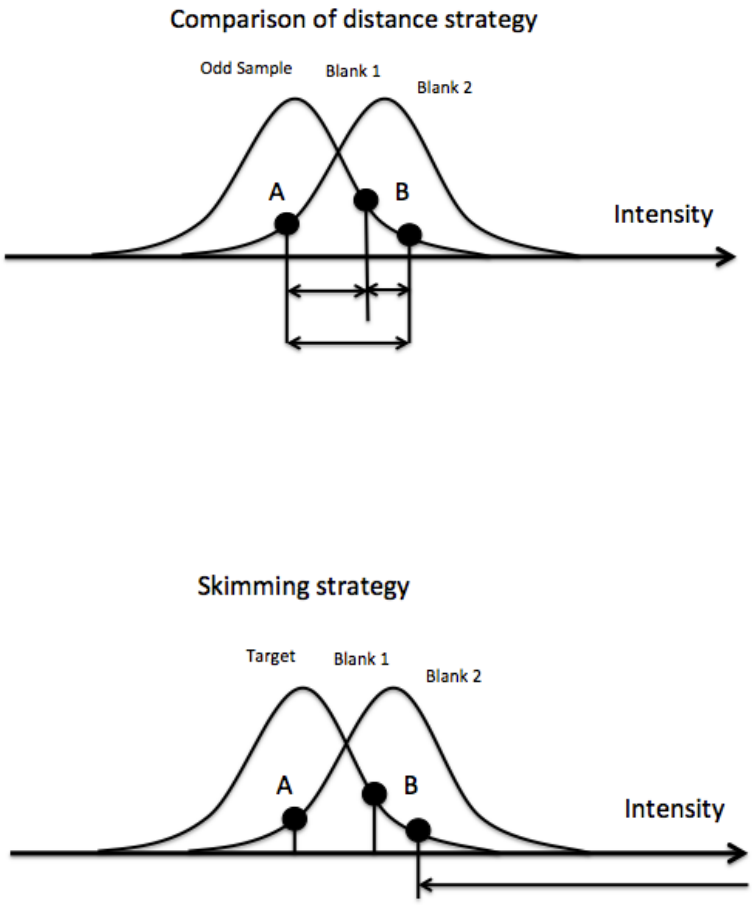

Figure 3.3 Scenario 3: With the same sensory evidence, comparison of distance strategy leads to a correct identification of the odd sample, whereas skimming strategy leads to a misidentification of the target sample.

3.2.2 Method of Data Evaluation Provided by ASTM E679

According to ASTM E679 (2011) all panelists are required to either (1) complete the evaluation of the series of concentration steps presented to them, or (2) reach a set in which the test sample is correctly identified, then continue to judge correctly in the higher concentration steps (ASTM E679, 2011, p.3). The best-estimate thresholds (BET) of individuals are calculated from the series of correct/incorrect responses produced separately by each panelist. This calculation method is based on the assumption that the threshold corresponds to a point of perceptual discontinuity (Peng et al 2012). The pattern of each panelist's judgments are recorded by writing a sequence containing '0' for an incorrect judgment or '+' for a correct judgment (ASTM E679, 2011). This sequence of judgments is presented in the order of the ascending concentrations of the added substance (ASTM E679, 2011). Calculating the BET of each panelist is by taking the geometric mean of the concentration steps at which the panelist's last incorrect judgment '0' occurred and the next higher concentration designated by a correct judgment '+' (ASTM E679, 2011). More specifically, the calculation method given by ASTM E679 requires two or more consistently correct judgments, i.e., since the last miss '0', by the panelist before the panelist's threshold can be calculated. ASTM E679 (2011) suggested, "If the concentration range has been correctly selected, the representation of the panelist's judgments should terminate with two or more consecutive plusses (+)" (p. 3). Group BET is calculated using the geometric means of the individual BETs (ASTM E679, 2011). **Table 3.1** is an example of the results of the individual BETs and the group BET calculated following the method given by ASTM E679 (2011). For example, the BET of panelist 01 is calculated using the geometric mean of the concentrations at the last incorrect judgment (60 mg/L) and the next correct judgment (120 mg/L). The formula for this calculation is: $\sqrt{60 * 120}$ = 84.8 mg/L. Then the threshold estimate is subsequently converted into the base 10 logarithm. Whereby, according to Fechner's Law of psychophysical method of quantifying sensation, the perceived intensity of a stimulus is not equal to the intensity of the physical stimulus, but proportional to the log of its physical intensity (Meilgaard et al 2007, p.50, Adler et al 2014). In the example shown in **Table 3.1**, the formula for calculating the group BET is: $10^{\mathbf{x(1.67)}}$=46.8 mg/L. The value 1.67 is the average value of $\Sigma\log_{10}$ = 26.73.

At times, when the chosen concentration steps cannot capture the panelist's threshold, ASTM E679 suggests that an endpoint rule can be applied to the data evaluation. For instance, when a panelist correctly identifies the odd sample in all concentration steps, the BET of this panelist is calculated as the geometric mean of the lowest concentration presented and the hypothetical one below it i.e., calculated by using the lowest concentration presented/the constant factor. On the other hand, if a panelist fails to identify the odd sample in all concentration steps, the

BET of this panelist is calculated as the geometric mean of the highest concentration presented and the next one higher, i.e., calculated by using the highest concentration presented multiplying the constant factor. ASTM justifies the use of the endpoint rule: “since the thresholds of the other panelists were within the presented scale range, his threshold should not be far away from the range if he belongs to the same statistical population” (ASTM E679, 2011, p. 5).

Table 3.1 BETs calculated based on an individual’s correct/incorrect response pattern and group BET calculated using the geometric means of the individual BETs (ASTM E679, 2011).

TABLE X1.1 Example of Difference Threshold for an Added Substance

Panelist	Judgments[A]											
	Concentrations of ethyl acetate presented, mg/L										Best-Estimate Threshold (BET)	
	10	15	20	30	40	60	80	120	160	240	Value	$\log_{10}$ of value
01	...	0	...	+	...	0	...	+	...	+	84.8	1.93
02	+	...	0	...	+	...	+	...	+	...	28.3	1.45
04	0	...	0	...	+	...	+	...	+	...	28.3	1.45
07	+	...	+	...	0	...	+	...	+	...	56.5	1.75
09	+	...	+	...	+	...	+	...	+	...	7.1	0.85
10	...	0	...	+	...	0	...	+	...	+	84.8	1.93
11	...	+	...	0	...	0	...	+	...	+	84.8	1.93
12	...	...	...	0	...	+	...	...	...	...	42.4	1.63
13	+	...	0	...	0	...	+	...	+	...	56.5	1.75
17	...	0	...	+	...	+	...	+	...	+	21.2	1.33
18	0	...	0	+	...	0	...	+	...	+	84.8	1.93
19	+	...	0	...	0	...	0	...	+	...	113	2.05
20	0	...	+	...	+	...	+	...	+	...	14.1	1.15
23	0	...	+	...	0	...	+	...	+	...	56.5	1.75
24	...	+	...	0	...	+	...	+	...	+	42.4	1.62
27	...	0	...	+	...	0	...	0	...	+	169.7	2.23
Group BET = geometric mean, mg/L ethyl acetate											$\Sigma\log_{10}$ →	26.73
											46.8 ←	1.67
Log Standard deviation												0.36

[A] “0” indicates that the panelist selected the wrong sample of the set of three. “+” indicates that the panelist selected the correct sample.

3.2.3 The Stopping Rules Associated with ASTM E679

In theory, to calculate the results from the concentration where the last misidentification ‘0’ occurred to the end of the concentration step, panelist’s judgments should consist of two or more consecutively correct judgments. Yet, in practice, it has been noted that the use of ASTM E679 requires making up another rule that specifies the number of correct responses from which the individual BET is calculated (Lawless and Heymann 2010, Peng et al 2012). The most commonly used one is referred to as the “Last Reversal” rule (Peng et al 2012). In this rule, the BET is calculated at the concentrations where the panelist’s judgment reversed from incorrect to correct for the last time. This is consistent with what is recommended by ASTM E679 (2011). In other cases, the BET is calculated at the concentrations at which the panelist’s judgment changed from incorrect to correct and followed by two or three consecutive correct judgments. Peng et al (2012) referred these two as the “Stop 2” and “Stop 3” rules. Lawless and Heymann (2010) explained that sometimes due to the possibility that panelists will become fatigued or adapted, experimenters may allow the panelist to stop tasting if since the last miss, there have

been three consecutive correct judgments. However, the authors suggested that invoking the stopping rule could increase the chance of getting Type I error (false positive).

Peng et al (2012) stated the two bias associated with the stopping rules were: (1) correct guesses (e.g., each 3-AFC trial has a 1/3 guessing probability) are mistaken as genuine detection in Stop 2 and Stop 3 rules, and (2) judgment errors (e.g., resulted from the use of a less optimal cognitive strategy) treated as detection failures in the Last Reversal rule. An example of the second bias, is illustrated by the upper figure in the scenario 2 of **Figure 3.2** where the panelist applied the comparison of distance strategy to the sensory evidence, thus misidentified the blank 1 as the odd sample. In this case, the result would be treated as an incorrect judgment '0', which means at this concentration step the panelist did not detect any difference between the control and the test sample. Yet, in fact, the panelist had detected the difference and if the skimming strategy were use, the panelist would have identified the target sample. In addition, Peng et al (2012) proposed that the choice of stopping rules could have strong impact on the individual BET, thus could result in inconsistent values of the group BET. Based on their experiment, the authors favoured the use of Last Reversal and Stop 3 rules and disputed the use of Stop 2 rule as it had given inconsistent BET values among the panelists' threshold measures.

3.2.4 The Use of ASTM E679 in the Present Study

In the present study, ASTM E679 is used to determine the effects of (1) tannin and (2) tannin-salivary protein interaction on the aroma perception (i.e., the intensity) of ethyl hexanoate in a wine-like solution. ASTM E679 (2011) defines a medium as "any material used to dissolve, disperse, or sorb odours or sapid material whose threshold is to be measured"; the blank sample as "a quantity of the medium containing no added odours"; and the test sample as " the medium to which an odorant has been added at a known concentration" (p.2). Therefore, unlike a typical detection threshold test where no aroma chemical is added into the blank control sample. For the present study, each test sample and blank control sample contained 300 μg/L of ethyl hexanoate. In addition, same as in Ross et al (2014) and Perry and Hayes (2016), each sensory test in the present study was considered as the triangle test and the data obtained from these test (i.e., the correct sensory response) were compared to the critical values for the triangle test.

3.2.5 Preliminary Experiments

A preliminary tannin concentration series (i.e., 2 g/L, 4 g/L, 8 g/L, 16 g/L and 32 g/L) was made with the objective of determining the final tannin concentration scale to be used in the main experiment. As recommended by ASTM E679, nine experienced wine sensory panelists were recruited for the preliminary experiment. Sensory judgements of the nine panelists were recorded and individual BETs and the group BET were calculated using the formulas provided by ASTM E679. For cases where the threshold was above or below the preliminary concentration range, the threshold was calculated by applying the endpoint rule (see section 3.2.3). **Table 3.2** shows the results from the first preliminary experiment. Each panelist's sensory threshold was calculated and converted into the base 10 logarithm as required by the method. The $\sum log_{10}$ value was calculated, i.e., 7.35, giving an average log_{10} value of 0.82, with a standard log_{10} deviation of 0.52. The group BET was calculated by converting the average log_{10} value, i.e., $10^{x(0.82)} = 6.61$.

Table 3.2 First preliminary experiment: sensory judgements by nine experienced wine sensory panelists on tannin samples containing ethyl hexanoate.

Panelists	Judgements at Five Tannin Concentrations (g/L) with Ethyl Hexanoate					Best-Estimate threshold (BET)	
	2	4	8	16	32	Value	Log 10 of Value
1	-	+	-	+	-	45	1.65
2	+	+	+	+	+	1.41	0.15
3	-	+	+	+	+	2.83	0.45
4	+	+	-	+	+	11.31	1.05
5	-	+	-	-	+	22.63	1.35
6	-	+	+	+	+	2.83	0.45
7	-	-	+	+	+	5.66	0.75
8	+	+	+	+	+	1.41	0.15
9	-	-	-	-	+	22.63	1.35
Group BET geometric mean						∑log 10 -->	7.35
						Average	0.82
						BET	6.61
						Std	0.56
Note: '+' represents a panelist selected the tannin test sample;' -' represents a panelist did not select the tannin test sample.							

Initially it seemed that at around 6.61 g/L of tannins, 50% of the panelists would be able to detect a change in the aroma intensity of ethyl hexanoate induced by tannin (i.e., tannin reduced the volatility of ethyl hexanoate). However, comments collected from the preliminary experiment showed that 6 panelists, who correctly chose the tannin test samples in the 4th and 5th sets, indicated that they detected a different aroma (i.e., a musky, oaky aroma) in the odd samples. Therefore, another preliminary experiment was carried out using the same wine-like solutions but without the addition of ethyl hexanoate. This was to determine whether or not grape extracted tannin solutions contained aromas that could interfere with the sensory experiment. **Table 3.3** shows the results from the second preliminary experiment. From the results it can be seen that at each tannin concentration (except for at 2 g/L of tannins), there were at least six panelists who correctly picked out the tannin test sample. Also from the

comments collected, most panelists indicated that they detected a different aroma in the test samples compared to the control samples. This strongly suggested that grape extracted tannins contributed some unexpected aromas, and that at around 4 g/L of tannins (i.e., the second lowest tannin concentration step), more than 50% of the panelists could detect those aromas in the test sample. This meant that aromas from grape extracted tannins would likely interfere with the aroma perception of tannin test samples (i.e., containing ethyl hexanoate), thus affecting the judgement of the panelists. In order to avoid this influence, it was decided to dearomatise the bulk tannin solution, from which the different tannin concentrations would be prepared.

Table 3.3 Second preliminary experiment: sensory judgements by nine experienced panelists on tannin samples with no ethyl hexanoate.

Panelists	Judgements at Five Tannin Concentrations (g/L) with no Ethyl Hexanoate					Best-Estimate threshold (BET)	
	2	4	8	16	32	Value	Log 10 of Value
1	+	+	+	+	+	1.41	0.15
2	+	+	+	+	+	1.41	0.15
3	+	+	+	+	+	1.41	0.15
4	-	+	-	+	+	11.3	1.05
5	-	-	-	-	+	22.6	1.35
6	-	+	+	+	+	2.83	0.45
7	-	-	+	+	+	5.66	0.75
8	+	+	+	+	+	1.41	0.15
9	-	-	+	-	+	22.6	1.35
Group BET geometric mean						∑log 10 -->	5.55
						Average	0.62
						BET	4.14
						Std	0.52
Note: '+' represents a panelist selected the tannin test sample;' -' represents a panelist did not select the tannin test sample.							

Dearomatisation

Dearomatisation was achieved using Lichrolut EN (Merck, New Zealand) with 4 g/L added to the tannin bulk solution (see section 3.3.4 for the dearomatisation procedure). Afterwards, two more preliminary experiments (i.e., the third and the fourth preliminary experiments) were carried out. The third preliminary experiment was to determine if the de-aromatisation was successful. In the third preliminary experiment, ethyl hexanoate was not added into the test and control samples. The results from the third preliminary experiment are shown in **Table 3.4**. In the first set, 4 panelists selected the tannin test sample; in the second set, 1 panelist selected the tannin test sample; in the third set, 3 panelists selected the tannin test sample; in the fourth set, 6 panelists selected the tannin test sample; and in the fifth set, 2 panelists selected the tannin test sample.

Table 3.4 Third preliminary experiment: sensory judgements on dearomatised tannin solutions with no ethyl hexanoate.

Panelists	Judgements at Five Tannin Concentrations (g/L) after De-aromatising with no Ethyl Hexanoate					Best-Estimate threshold (BET)	
	2	4	8	16	32	Value	Log 10 of Value
1	-	+	+	+	-	45	1.65
2	+	-	+	+	+	5.66	0.75
3	-	-	-	+	-	45	1.65
4	+	-	-	-	-	45	1.65
5	+	-	-	+	-	45	1.65
6	+	-	-	+	+	11.31	1.05
7	-	-	-	-	-	45	1.65
8	-	-	+	-	-	45	1.65
9	-	-	-	+	-	45	1.65
Group BET geometric mean						∑log 10 -->	13.35
						Average	1.48
						BET	30
						Std	0.339
Note: '+' represents a panelist selected the tannin test sample;' -' represents a panelist did not select the tannin test sample.							

In **Figure 3.4**, it can be seen that using dearomatised tannin samples, the proportion of panelists selecting the tannin sample was reduced at all concentrations. In addition, at 4 g/L, 8 g/L and 32 g/L tannin concentrations, the rate of panelists selecting the tannin sample was lower or equal to the chance of guessing (33%) given in the 3-AFC and triangle tests. In terms of the comments collected from the 16 g/L tannin sample, five out of the six panelists indicated that compared to the control samples the odd sample had the same aroma but different aroma intensity. This was not expected as the control samples had no aroma compound and the only smell from the control samples would be the smell of the 12% ethanol. Also, if the dearomatised tannin test sample still had residual aromas, we would expect these panelists to indicate that the test sample had a different aroma than the controls not only at 16 g/L of tannins, but also at 32 g/L of tannins.

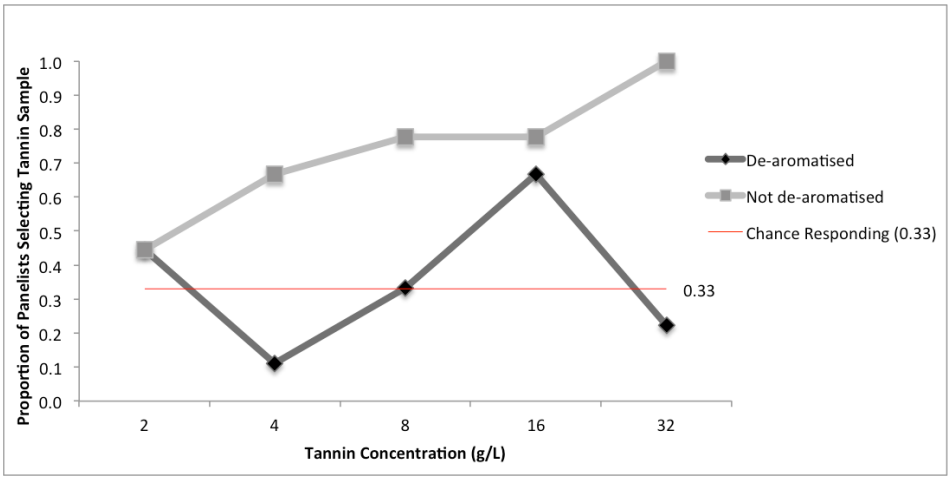

Figure 3.4 Proportion of panelists selecting the tannin test sample with no ethyl hexanoate at each tannin concentration before and after dearomatisation.

In order to confirm that at 16 g/L of tannins, the dearomatised tannin solution was devoid of aromas, another triangle test was carried out at this tannin concentration. This time the nine

panelists were asked to evaluate two sets of three samples that had two control samples and the one tannin sample (i.e., 16 g/L of tannins) presented in random orders (i.e., AAB, ABA, BAA). The results in **Table 3.5** demonstrate that the detection rate of panelists selecting the tannin sample was 0.28, which was lower than the chance of guessing (i.e., 0.33). Therefore, the results from these preliminary experiments suggest that after dearomatisation, panelists could not detect any significant aroma difference between the control solution and the dearomatised tannin solutions at the five chosen concentrations.

Table 3.5 Judgements of nine panelists on dearomatised tannin solution with 16 g/L of tannins.

Panelist	1	2	3	4	5	6	7	8	9
Judgement at 1st set	+	-	+	-	-	-	+	-	+
Judgement at 2nd set	-	-	-	-	-	-	-	+	-
The detection rate of panelists selecting the tannin test sample averaged across the two triangle tests							0.28		
Note: '+' represents a panelist selected the tannin test sample;' -' represents a panelist did not select the tannin test sample.									

The preliminary tannin concentration series (i.e., 2 g/L, 4 g/L, 8 g/L, 16 g/L and 32 g/L) was then made using dearomatised tannin bulk solution to determine the final tannin concentration scale. **Table 3.6** shows the results from the final preliminary experiment. Each panelist's sensory threshold was calculated and converted into the base 10 logarithm as performed in the first preliminary experiment. The $\sum log_{10}$ value was calculated, i.e., 6.45, giving an average log_{10} value of 0.72, with a standard log_{10} deviation of 0.38. The group BET was calculated by converting the average log_{10} value, i.e., $10^{x(0.72)}$ = 5.29. We can see that at 16 g/L and 32 g/L of tannins, all nine panelists had selected the tannin test sample. Also, at 2 g/L of tannins, there were five panelists that had selected the tannin test sample. Based on the method provided by ASTM E679 (2011, *pp* 2), in which the final concentration scale should begin well below the concentration at which the most sensitive panelist is able to detect the targeted sample, and end at (or above) the concentration at which all panelists can detect the targeted sample. The final concentration scale for the main study was chosen to be: 0.6, 1.8, 5.4, and 16.2 g/L of tannins, giving 4 test samples. Any two adjacent tannin concentration steps were separated by a constant factor of 3.

Table 3.6 After tannin dearomatisation, the sensory judgements by nine experienced wine sensory panelists of samples.

Panelists	Judgements at Five Tannin Concentrations (g/L) with Ethyl Hexanoate					Best-Estimate threshold (BET)	
	2	4	8	16	32	Value	Log 10 of Value
1	-	-	+	+	+	5.65	0.75
2	+	+	+	+	+	1.41	0.15
3	+	-	-	+	+	11.3	1.05
4	-	+	+	+	+	2.83	0.45
5	+	+	-	+	+	11.3	1.05
6	-	-	+	+	+	5.65	0.75
7	-	+	-	+	+	11.3	1.05
8	+	+	+	+	+	1.41	0.15
9	+	-	-	+	+	11.3	1.05
Group BET geometric mean						∑log 10 -->	6.45
						Average	0.72
						BET	5.29
						Std	0.38
Note: '+' represents a panelist selected the tannin test sample;' -' represents a panelist did not select the tannin test sample.							

3.3 Reagents and Sample Preparation

3.3.1 Chemicals

Ethyl hexanoate (≥ 99%) was purchased from Sigma-Aldrich (New Jersey, USA). Analytical reagent grade (AR) ethanol (99.8%) and sodium hydroxide of 1 M were purchased from Fisher Scientific UK Limited (Leicestershire, UK). Tartaric acid (≥99%) was purchased from ACROS Organics (New Jersey, USA). pH buffer solutions (pH 4 and pH 7) were purchased from Fisher Scientific UK Limited (Leicestershire, UK). Commercial extracts of grape seed and skin tannins were purchased from IOC Essential, Institut Oenologique de Champagne, Epernay, France. Porcine gastric mucin (PGM, type III)) was purchased from Sigma-Aldrich (St. Louis, MO, USA). $NaHCO_3$ (99.7-100.3%) AnalaR NORMAPUR was purchased from VWR Chemicals (Germany). $CaCl \cdot 2H_2O$, $K_2HPO_4 \cdot 3H_2O$, NaCl, and KCl were purchased from Sigma-Aldrich (Victoria, AUS). Distilled water was purified by the Thermo Scientific™ Barnstead™ Pacifi™ RO Water Purification System (Germany). Deionised water was purified by Thermo Scientific™ Barnstead™ GenPure™ Pro Water Purification System (Germany).

3.3.2 Preparation of the Artificial Saliva Solution

As the collection of human saliva for experimental purposes can be tedious and unpleasant, the use of formulated artificial saliva has been used or examined as the alternative in several studies (van Ruth and Roozen 2000, Friel and Taylor 2001, Genovese et al 2009, Mitropoulou et al 2011). In artificial saliva, the protein components (i.e., amylase and mucin) are often substituted by those from the nonhuman sources, which are more abundant and economical (Friel and

Taylor 2001). van Ruth and Roozen (2000) studied the impact of artificial and natural saliva on the aroma release of six compounds and found no significant difference in the aroma release between the use of artificial and natural saliva. Friel and Taylor (2001) suggested that using artificial saliva with substituted protein sources seemed to be an acceptable alternative to using human saliva. Of note, Genovese et al (2009) reported a greater decrease of headspace concentration of esters and acetates in wine with human saliva compared with artificial saliva. The authors explained that this difference could be due to both the enzymes (such as esterases) and mucin in human saliva.

One of the aims of the present study was to determine the influence of salivary protein on the volatility of ethyl hexanoate in a wine-like solution containing tannin, therefore using artificial saliva with mucin was suited to the purpose. According to Friel and Taylor (2001), salts may modify the number of available binding sites of mucin and may result in formation of hydrophobic inclusion sites that can help trap volatiles within the solution structure. Artificial saliva was prepared by using porcine gastric mucin (PGM, type III, Sigma-Aldrich) of 2.160 g mixed with $NaHCO_3$ (5.208 g), $K_2HPO_4 \cdot 3H_2O$ (1.372 g), NaCl (0.880 g), KCl (0.4481 g), $CaCl \cdot 2H_2O$ (1.7645 g) and distilled water in a 1 L volumetric flask. This formula was found in Mitropoulou et al (2011). Afterwards the saliva is centrifuged at 10000 rpm for 10 min to remove any undissolved material. The artificial saliva was stored at 4 ^{o}C until use.

3.3.3 Preparation of the Aroma Compound for the Experiment

Table 3.7 shows the perception threshold and odour descriptions of ethyl hexanoate. Several studies have reported and/or used the concentration of ethyl hexanoate found in different wines (Fang and Qian 2006, Escudero et al 2007, Schreier 1980). In the present study, the targeted concentration of ethyl hexanoate in the control and tannin solutions was 300 μg/L.

Table 3.7 The perception threshold and odour descriptions of ethyl hexanoate.

Aroma Compound	Unit	Perception Threshold	Odour Description	Concentration Found in Literature		
Ethyl hexanoate	μg/L	14[a]	fruity; strawberry	179 -296 [b]	29-227[c]	200-470[d]

a Escudero et al (2007), b Fang and Qian (2006), c Escudero et al (2007), d Schreier (1980).

Ethyl Hexanoate Primary Standard for Sensory Experiment

Prior to use, a primary standard of ethyl hexanoate was prepared (**Table 3.8**). The method of preparing the primary standard solution was by direct weighing the pure reagent (99%) (i.e., 'weight out' in **Table 3.8**) and adding the solvent (i.e., AR ethanol of 99.8%) to make up a 50 mL solution in a volumetric flask. The formula for calculating the targeted concentration of primary standard solution was: [Targeted amount to weigh out (g) x stock solution purity (%)]/0.05(L). Thus, the targeted primary solution concentration of ethyl hexanoate was calculated as: (0.20 x 0.99)/0.05 = 3.96 g/L. The actual concentration of the primary standard solution was: (0.2002 g x 0.99)/0.05(L) = 3.964 g/L. The volumetric flask was rinsed with the AR ethanol and allowed to dry before use. Weighing was carried out using the Mettler-Toledo AG104 scale in a fume cupboard and recorded to the 4th decimal place. The primary solution of ethyl hexanoate was transferred into a 50 mL amber vial and stored in the freezer at -20 ^{o}C until use.

Table 3.8 Preparation of Primary Standard Solution of Ethyl Hexanoate for Sensory Experiment.

Aroma Compounds	Stok Solution Purity	Neat Concentration	Weight out (g)	Primary Standard Concentration (g/L)
Ethyl hexanoate	99%	99 g/ 100 g	0.2002	3.9640

Note: Primary standard solution was made up to 50 mL in AR ethanol of 99.8%.

Ethyl Hexanoate Primary Standard for Instrumental Experiment

The ethyl hexanoate primary standard for the instrumental analysis was prepared using the same procedure as for the sensory experiment but with HPLC ethanol of 99.9% (v/v). The actual concentration of the primary standard solution was: (0.2021 g x 0.99)/0.05(L) = 4.0016 g/L (**Table 3.9**). The volumetric flask was rinsed with the HPLC ethanol and allowed to dry before use. Weighing was carried out using the Mettler-Toledo AG104 scale in a fume cupboard and recorded to the 4th decimal place. The primary solution of ethyl hexanoate was transferred into a 50 mL amber vial and stored in the freezer at -20 ^{o}C until use.

Table 3.9 Preparation of Primary Standard Solution of Ethyl Hexanoate for Instrumental Analysis.

Aroma Compounds	Stock Solution Purity	Neat Concentration	Weight out (g)	Primary Standard Concentration (g/L)
Ethyl hexanoate	99%	99 g/ 100 g	0.2021	4.0016

Note: Primary standard solution was made up to 50 mL in AR ethanol of 99.8%.

3.3.4 Preparations for the Wine-like Solutions for Sensory Experiment

Preliminary Experiments

A control solution of 2 L was prepared in a volumetric flask using 5 g/L of tartaric acid, 240 mL of AR graded ethanol (99.8%) and distilled water to obtain an ethanol concentration of approximately 12% vol (v/v). The formula for calculating the ethanol dilution was: the required volume of pure AR ethanol (L) = [(desired strength (i.e. 12% vol) x targeted volume (i.e., 2000 mL)] /[pure AR ethanol content (i.e., 99.8%)]. The control solution was mixed thoroughly and left stirring for 30 minutes. Afterwards, the pH was adjusted to 3.5 by titration (2.6mL) with 10 M sodium hydroxide. The volume of sodium hydroxide used to adjust the pH was small and should not cause any significant dilution to the other components of the control solution. The control solution was divided into two equal portions. To the first portion of 1 L, 75.68 μL of ethyl hexanoate primary solution was added, giving 300 μg/L of ethyl hexanoate in the control solution. This required amount (μL) of ethyl hexanoate primary standard was calculated using: [(300 μg/L x 1 L)/(3.9640 x 1000000 μg/L)] x 1000000 (μL/L). The second portion of 1 L was kept without the addition of ethyl hexanoate and was used in the third preliminary experiment.

The tannin bulk solution (i.e., with 32 g/L of tannins) was prepared in a volumetric flask using 32g of tannins (4:1, seed to skin tannins) and 120 mL of AR graded ethanol of 99.8% and distilled water to obtain an ethanol concentration of approximately 12% vol (v/v). The solution was stirred for 30 minutes under nitrogen in the volumetric flask. It was then dearomatised using 4 g/L of Lichrolut EN (Merck, New Zealand) as the dearomatisation agent and flushed with nitrogen and left stirring for 18 hours. Afterwards, tannin bulk solution was filtered using

Whatman ® Grade 4 filter papers. The method for dearomatisation was described in Tomasino (2011), *pp.* 105.

The 1 L of 12% vol (v/v) ethanol base solution was prepared in a volumetric flask using 120 mL of AR graded ethanol of 99.8% and distilled water.

The T32 tannin solution, containing 5 g/L tartaric acid, 12% ethanol and 32 g/L of tannins, was prepared in a 500 mL volumetric flask using 2.5 g of tartaric acid and the tannin bulk solution. The pH of T32 solution was adjusted to 3.5 by titration (1.8 mL) with 10 M sodium hydroxide. Afterwards, 200 mL of the T32 solution was transferred into a glass bottle and then spiked with 15.14 μL of ethyl hexanoate primary solution. This required amount of ethyl hexanoate primary standard was calculated using: [(300 μg/L x 200 mL)/(3.9640 x 1000000 μg/L)] x 1000000 (μL/L).The rest of the T32 solution was kept without the addition of ethyl hexanoate and was used to examine the effectiveness of dearomatisation.

The T16 tannin solution, containing 5 g/L tartaric acid, 12% ethanol and 16 g/L of tannins, was made in a 500 mL volumetric flask using 2.5 g of tartaric acid, 250 mL of the tannin bulk solution and the 12% ethanol base solution. The required volume of tannin bulk solution was calculated using: [Targeted tannin concentration (i.e., 16 g/L) x targeted volume (i.e., 500 mL)]/[Tannin concentration of the tannin bulk solution (i.e., 32 g/L)]. The pH of T16 solution was adjusted to 3.5 by titration (1.8 mL) with 10 M sodium hydroxide. Afterwards, 200 mL of the T16 solution (i.e., T16a) was transferred into a glass bottle and then spiked with 15.14 μL of ethyl hexanoate primary solution (calculated using the same formula as described previously). The rest of the T16 solution (i.e., T16b) was kept without the addition of ethyl hexanoate.

T8 tannin solution, containing 5 g/L tartaric acid, 12% ethanol and 8 g/L of tannins, was made in a 500 mL volumetric flask using 2.5 g of tartaric acid and 125 mL of the tannin bulk solution and the 12% ethanol base solution. The pH of T8 solution was adjusted to 3.5 by titration (1.8 mL) with 10 M sodium hydroxide. Afterwards, 200 mL of the T8 solution (i.e., T8a) was transferred into a glass bottle and then spiked with 15.14 μL of ethyl hexanoate primary solution. The rest of the T8 solution (i.e., T8b) was kept without the addition of ethyl hexanoate.

T4 tannin solution, containing 5 g/L tartaric acid, 12% ethanol and 4 g/L of tannins, was made in a 500 mL volumetric flask using 2.5 g of tartaric acid and 62.5 mL of the tannin bulk solution and the 12% ethanol base solution. The pH of T4 solution was adjusted to 3.5 by titration (1.6 mL) with 10 M sodium hydroxide. Afterwards, 200 mL of the T4 solution (i.e., T4a) was transferred into a glass bottle and then spiked with 15.14 μL of ethyl hexanoate primary solution. The rest of the T4 solution (i.e., T4b) was kept without the addition of ethyl hexanoate.

T2 tannin solution, containing 5 g/L tartaric acid, 12% ethanol and 2 g/L of tannins, was made in a 500 mL volumetric flask using 2.5 g of tartaric acid and 31.25 mL of tannin bulk solution and the 12% ethanol base solution. The pH of T2 solution was adjusted to 3.5 by titration (1.6 mL) with 10 M sodium hydroxide. Afterwards, 200 mL of the T2 solution (i.e., T2a) was transferred into a glass bottle and then spiked with 15.14 μL of ethyl hexanoate primary solution. The rest of the T2 solution (i.e., T2b) was kept without the addition of ethyl hexanoate.

Comparing to the volume of tannin solution, the amount of 10 M sodium hydroxide used in pH adjustment (i.e., 1.6 ~ 1.8 mL) was very small. This amount of sodium hydroxide would not cause significant dilution to the other components of the tannin solution. Also the variations in the amount of sodium hydroxide used in pH adjustment across the five tannin solutions were very small, which would not result in significant differences in the concentrations of ethanol and tartaric acid between the five tannin solutions. This is illustrated in **Table 3.10**. and **Table 3.11**.

Table 3.10 An example of the influence of pH adjustment on the concentrations of ethanol, tannin, and tartaric acid in T32 tannin solution.

T32 tannin solution	Tannin Concentration	Tartaric acid	Ethanol %
Before pH adjustment	32 g/L	5 g/L	12 %
After pH adjustment	31.9 g/L	4.98 g/L	11.96 %

*** The concentration after pH adjustment was calculated using: (the concentration before pH adjustment) x (500/501.8).**

Table 3.11 The influence of pH adjustment on the concentrations of ethanol and tartaric acid across the five tannin solutions.

After pH adjustment	T32	T16	T8	T4	T2
Ethanol %	11.956 %	11.956 %	11.956%	11.961%	11.961%
Tartaric acid	4.982 g/L	4.982 g/L	4.982 g/L	4.984 g/L	4.984 g/L

* **The concentration after pH adjustment was calculated using: (the concentration before pH adjustment) x [500 mL / (500 + the volume of sodium hydroxide used) mL].**

Main Sensory Experiments

In the human mouth, the average ratio of liquid food/saliva had previously been determined to be 5:1 w/v (Genovese et al 2009). The test samples containing the control wine-like solution or

the wine-like solution with tannins were prepared by adding 20 mL of the control or the tannin solutions into the wine glass. After that, 4 mL of artificial saliva was added. This addition of artificial saliva would dilute the concentrations of ethanol, tannin, tartaric acid and ethyl hexanoate in the samples. Therefore, considering the dilution effect, the concentrations of ethanol, tannin, tartaric acid and ethyl hexanoate in the samples for the main experiment were increased by 1.2 times (i.e., 24/20) compared to those for the preliminary experiments. This allowed sample dilution to be canceled out, simplifying the procedure.

Table 3.12 Adjusted concentrations of ethanol, tannin, tartaric acid and ethyl hexanoate in the control and tannin solutions.

Targeted tannin concentration in the test samples	0.6 g/L	1.8 g/L	5.4 g/L	16.25 g/L
Tannin concentration in the tannin solutions	0.72 g/L	2.16 g/L	6.48 g/L	19.5 g/L
Targeted ethanol % in the test samples	12 %			
Ethanol % in the control and tannin solutions	14.4%			
Targeted tartaric acid concentration in the test samples	5 g/L			
Tartaric acid concentration in the control and tannin solutions	6 g/L			
Targeted ethyl hexanoate in the test samples	300 μg/L			
Ethyl hexanoate in the control and tannin solutions	360 μg/L			

The control solution was prepared in a 5 L volumetric flask using 6 g/L of tartaric acid, 720 mL of AR graded ethanol (99.8%) and distilled water to obtain an ethanol concentration of approximately 14.4% vol (v/v). The control solution was mixed thoroughly and left stirring for 30 minutes. Afterwards, pH was adjusted to 3.5 by titration (7.6 mL) with 10 M sodium hydroxide. A total of 15 L of control solution was prepared and stored in a plastic container. After that, 1.3623 mL of ethyl hexanoate primary solution was added into the 15 L of control solution, giving 360 μg/L of ethyl hexanoate in the control wine-like solution. This required amount of ethyl hexanoate primary standard was calculated using: [(360 μg/L x 15 L)/(3.9640 x 1000000 μg/L)] x1000 (mL/L).

The tannin bulk solution (i.e., 19.5 g/L of tannins) was prepared in a 1 L volumetric flask using 19.5 g of tannins (4:1, seed to skin tannins) and 144 mL of AR graded ethanol (99.8%) and distilled water to obtain an ethanol concentration of approximately 14.4% vol (v/v). The solution was stirred for 30 minutes under nitrogen in the volumetric flask. A total of 3 L of tannin bulk solution was prepared. It was then dearomatised using 4 g/L of Lichrolut EN (Merck, New Zealand) as the dearomatisation agent and flushed with nitrogen and left stirring for 18 hours. Afterwards, tannin bulk solution was filtered using Whatman ® Grade 4 filter papers.

The 5L of 14.4% vol (v/v) ethanol base solution was made in a 5 L volumetric flask using 720 mL of AR graded ethanol (99.8%) and distilled water.

The T19.5 tannin solution, containing 6 g/L tartaric acid, 14.4% ethanol and 19.5 g/L of tannins, was prepared in a 2 L volumetric flask using 12 g of tartaric acid and the tannin bulk solution. The pH of T19.5 solution was adjusted to 3.5 by titration (5.6 mL) with 10 M sodium hydroxide.

T6.48 tannin solution, containing 6 g/L tartaric acid, 14.4% ethanol and 6.48 g/L of tannins, was prepared in a 2 L volumetric flask using 12 g of tartaric acid, 664.6mL of the tannin bulk solution and the 14.4% ethanol base solution. The required volume of tannin bulk solution was calculated using: [Targeted tannin concentration (i.e., 6.48 g/L) x targeted volume (i.e., 2 L)]/[Tannin concentration of the tannin bulk solution (i.e., 19.5 g/L)]. The pH of T6.48 solution was adjusted to 3.5 by titration (5.6 mL) with 10 M sodium hydroxide.

T2.16 tannin solution, containing 6 g/L tartaric acid, 14.4% ethanol and 2.16 g/L of tannins, was prepared in a 2 L volumetric flask using 12 g of tartaric acid, 221.6 mL of the tannin bulk solution and the 14.4% ethanol base solution. The pH of T2.16 solution was adjusted to 3.5 by titration (6.0 mL) with 10 M sodium hydroxide.

T0.72 tannin solution, containing 6 g/L tartaric acid, 14.4% ethanol and 0.72 g/L of tannins, was prepared in a 2 L volumetric flask using 12 g of tartaric acid, 61.8 mL of the tannin bulk solution and the 14.4% ethanol base solution. The pH of T0.72 solution was adjusted to 3.5 by titration (6.2 mL) with 10 M sodium hydroxide.

Afterwards, each solution was spiked with 0.1816 mL of the ethyl hexanoate primary solution, giving 360 μg/L of ethyl hexanoate in the solution. This required volume of the ethyl hexanoate primary standard was calculated using: (360 μg/L x 2 L)/(3.9640 x 1000000 μg/L) x 1000 (mL/L). Each solution was then divided into two equal portions of 1 L. 1 L was for the first sensory experiment on day 1, the other was for the second sensory experiment on day 2.

Once again, comparing to the volume of tannin solution, the volume of 10 M sodium hydroxide used (5.6 ~ 6.2 mL) to adjust the pH would not cause any significant dilution to the other components of the tannin solution. The variations in the volume of sodium hydroxide used in pH adjustment over the four tannin solutions were relatively small, which would not result in any significant difference in the concentrations of ethanol and tartaric acid between the four tannin solutions (**Table 3.13**). The concentration of each component after the pH adjustment was calculated using: (the concentration before the pH adjustment) x [1000 mL / (1000 + the volume of sodium hydroxide used)].

Table 3.13 The influence of pH adjustment on the concentrations of tannin, ethanol and tartaric acid across the control and four tannin solutions prepared for sensory experiment.

After pH adjustment	**T19.5**	**T6.48**	**T2.16**	**T0.72**	**Control**
Tannin Concentration	19.45 g/L	6.46 g/L	2.15 g/L	0.71 g/L	0 g/L
Ethanol %	14.36 %	14.36%	14.36%	14.36%	14.38%
Tartaric acid	5.98 g/L	5.98 g/L	5.98 g/L	5.98 g/L	5.99 g/L

*** The concentration after pH adjustment was calculated using: (the concentration before pH adjustment) x [200 mL / (200 + the volume of sodium hydroxide used) mL].**

3.3.5 Preparations for the Wine-like Solutions for Instrumental Analysis

The control solution was prepared in a 100 mL volumetric flask using 6 g/L of tartaric acid, 14.4 mL of HPLC ethanol (99.9%) and deionised water (18.12 MΩ·cm) to obtain an ethanol concentration of approximately 14.4% vol (v/v). The control solution was mixed thoroughly and left stirring for 30 minutes. Afterwards, the pH was adjusted to 3.5 by titration (0.4 mL) with 10 M sodium hydroxide. The solution was then transferred into a glass bottle and spiked with 8.996 μL of the ethyl hexanoate primary standard, giving 360 μg/L of ethyl hexanoate in the solution. This required amount of ethyl hexanoate primary standard was calculated using: [(360 μg/L x 100 mL)/(4.0016 x 1000000 μg/L)] x 1000 (μL/mL).

The tannin bulk solution (i.e., 19.5 g/L of tannins) was prepared in a 200 mL volumetric flask using 3.9 g of tannins (4:1, seed to skin tannins) and 28.8 mL of HPLC ethanol of 99.9% and deionised water (18.12 MΩ·cm) to obtain an ethanol concentration of approximately 14.4% vol (v/v). The solution was stirred for 30 minutes under nitrogen in the volumetric flask. It was then dearomatised using 0.8 g (i.e., 4 g/L) of Lichrolut EN (Merck, New Zealand) as the dearomatisation agent and flushed with nitrogen and left stirring for 18 hours. Afterwards, tannin bulk solution was filtered using Whatman ® Grade 4 filter papers.

The T19.5 tannin solution, containing 6 g/L tartaric acid, 14.4% ethanol and 19.5 g/L of tannins, was prepared in a 100 mL volumetric flask using 0.6 g of tartaric acid and the tannin bulk solution. The pH of T19.5 solution was adjusted to 3.5 by titration (0.4 mL) with 10 M sodium hydroxide.

T6.48 tannin solution, containing 6 g/L tartaric acid, 14.4% ethanol and 6.48 g/L of tannins, was prepared in a 100 mL volumetric flask using 0.6 g of tartaric acid, 33.2 mL of the tannin bulk solution and the 14.4% ethanol base solution. The required volume of tannin bulk solution was calculated using: [Targeted tannin concentration (i.e., 6.48 g/L) x targeted volume (i.e., 100 mL)]/[Tannin concentration of the tannin bulk solution (i.e., 19.5 g/L)]. The pH of T6.48 solution was adjusted to 3.5 by titration (0.4 mL) with 10 M sodium hydroxide.

T2.16 tannin solution, containing 6 g/L tartaric acid, 14.4% ethanol and 2.16 g/L of tannins, was prepared in a 100 mL volumetric flask using 0.6 g of tartaric acid, 11.1 mL of the tannin bulk solution and the 14.4% ethanol base solution. The pH of T2.16 solution was adjusted to 3.5 by titration (0.4 mL) with 10 M sodium hydroxide.

T0.72 tannin solution, containing 6 g/L tartaric acid, 14.4% ethanol and 0.72 g/L of tannins, was prepared in a 100 mL volumetric flask using 0.6 g of tartaric acid, 3.69 mL of the tannin bulk solution and the 14.4% ethanol base solution. The pH of T0.72 solution was adjusted to 3.5 by titration (0.4 mL) with 10 M sodium hydroxide.

Afterwards, each solution was then transferred into a glass bottle and spiked with 8.996 μL of the ethyl hexanoate primary standard, giving 360 μg/L of ethyl hexanoate in the solution. This required amount of ethyl hexanoate primary standard was calculated using: (360 μg/L x 100 mL)/(4.0016 x 1000000 μg/L) x 1000 (μL/mL).

After pH adjustment, the concentration variations of ethanol, tartaric acid and tannins between each solution are shown in **Table 3.14**.

Table 3.14 The influence of pH adjustment on the concentrations of tannin, ethanol and tartaric acid across the control and four tannin solutions prepared for analytical analysis.

After pH adjustment	**T19.5**	**T6.48**	**T2.16**	**T0.72**	**Control**
Tannin Concentration	19.42 g/L	6.45 g/L	2.15 g/L	0.72 g/L	0 g/L
Ethanol %	14.34 %	14.34%	14.36%	14.34%	14.34%
Tartaric acid	5.98 g/L	5.98 g/L	5.98 g/L	5.98 g/L	5.98 g/L

*** The concentration after pH adjustment was calculated using: (the concentration before pH adjustment) x [100 mL / (100 + the volume of sodium hydroxide used) mL].**

3.4 Method for Instrumental Analysis

In the recent decade, the technique that is called Headspace-Solid Phase Micro-Extraction-Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS) has been frequently used in wine volatile compound analysis. This technique with advantages of solvent free and non-invasive for the samples is a fast and effective way to identify and quantify the volatile organic compounds present at trace levels in wine. It consists of two processes: 1) extraction of the volatile compounds in the headspace (HS-SPME), and 2) obtaining qualitative information on the volatile compounds (GC-MS). HS-SPME is based on a consequent transfer of volatile compounds from the matrix of a sealed sample to the headspace and the volatile compounds are then adsorbed onto a specific SPME fiber that is inside of a hypodermic needle (Sagandykova et al 2017). This is then followed by a rapid thermal desorption of the volatile compounds in the injection port of a gas chromatograph-mass spectrometer (GC-MS) (Sagandykova et al 2017). It has been reported that HS-SPME allows the extraction of a large number of molecules with low detection limits (Rebière et al 2010, Chin et al 2012). In addition, this method combines sampling, extraction and concentration into one single step with no solvent needed during the process (Rodrigues et al 2008, Chin et al 2012). Once entering the GC, the volatile compounds are separated within the GC's capillary column in a chromatographic run before entering the MS for qualitative and/or quantitative analysis. For the present study, HS-SPME-GC-MS was used to determine the concentration of ethyl hexanoate in the headspace of the test samples.

3.4.1 Static Headspace-SPME Sampling Procedure

Literature suggested that the effect of saliva on wine aroma release was more evident when using static than dynamic headspace sampling procedure (Munoz-Gonzalez et al 2014). Therefore, in the present study, static headspace-SPME sampling procedure was used. Prior to the experiment, calibration was carried out to test the SPME extraction as well as the linearity and reproducibility of the sampling procedure. External HS-SPME-GC-MS standards were prepared with 12 % HPLC ethanol, 5 g/L tartaric acid and ethyl hexanoate (i.e., ranged from 30 to 500 μg/L). Regarding the choice of SPME fiber, a DVB/CAR/PDMS (divinylbenzene/ carboxen / polydimethylsiloxane, 50/30 μm thickness and 2 cm length) coated SPME fiber was used in headspace extraction. This type of fiber has been the popular choice in analytes extraction (Rebière et al 2010, Gamero et al 2013, Sagandykova et al 2017). It has been demonstrated that non-polar compounds were more effectively adsorbed and desorbed on the PDMS material, while polar compounds were more effectively adsorbed and desorbed on the DVB/PDMS

material (Rebière et al 2010, Chin et al 2012). In addition, Rebière et al (2010) found that the CAR/DVB/PDMS fiber provided good extraction capacity for a good range of compounds with molecular weight from 40-275 (g/mol). The average molecular weight of ethyl hexanoate is 144.21 g/mol. Therefore the use of CAR/DVB/PDMS fiber would provide a good extraction of the aroma compound examined in this study.

Sample preparation involved pipetting 8.33mL of model wine into 20 ml SPME sample vials along with 1.67mL of either deionised water or artificial saliva solution, 40µL of internal standard solution was then added immediately prior to capping. After adding the 1.67 mL of deionised water or the artificial saliva, the final concentrations of ethanol, tartaric acid and tannins of each test sample are showing in **Table 3.15.** The vials were immediately closed with a screw cap and polytetrafluoroethylene (PTFE)/ silicone septum and were placed in the waiting tray of an automatic headspace sampling device held at 8 ^{o}C. Before taken for SPME sampling, test samples were incubated for 30 minutes at 20 ^{o}C, allowing the equilibration of the headspace, and were then agitated for the last 10 minutes. The enclosed headspace was then exposed to a 2 cm long DVB/CAR/PDMS combination SPME fiber (p/n 57348-U, 50/30 µm thickness, 24 gauge, Supelco Bellefonte, PA, USA, through Sigma- Aldrich, Australia) for an extraction time of 2 minutes. During this exposure period the headspace volatiles, were adsorbed onto the fiber. During the headspace extraction, the temperature of the samples was maintained at 20 ^{o}C. The 2-minute extraction time used for the headspace extraction was described in literature (Munoz-Gonzalez, 2014). It was to simulate the time needed for the "swallow breath" to reach the maximum capacity after swallowing in the human mouth. Swallow breath is directly associated with retronasal olfaction where the aroma compounds are transferred from the mouth to the nasal cavity. Buettner and Schieberle (2000) studied the in-mouth equilibration time on the total amount of ethyl butanoate exhaled through nose after swallowing of a model solution and found that the maximum amount of ethyl butanoate exhaled was captured at around 2 minutes. In addition, Linforth et al (2002) demonstrated that the volume of swallow breath exhaled from one nostril reached maximum at very close to 2 minutes after swallowing a solution of cymene in water.

Table 3.15 After adding the 1.67 mL of deionised water or artificial saliva, the final concentrations of tannin, ethanol and tartaric acid in the control and the four tannin samples prepared for analytical analysis**.**

Test samples	T16.2	T5.4	T1.8	T0.6	Control
Tannin concentration after pH adjustment	19.42 g/L	6.45 g/L	2.15 g/L	0.72 g/L	0 g/L
Final tannin concentrations	16.18 g/L	5.38 g/L	1.79 g/L	0.6 g/L	0 g/L
Ethanol concentration after pH adjustment	14.34 %	14.34%	14.36%	14.34%	14.34%
Final ethanol concentration	11.95 %	11.95 %	11.97 %	11.95 %	11.95 %
Tartaric acid concentration after pH adjustment	5.98 g/L	5.98 g/L	5.98 g/L	5.98 g/L	5.98 g/L
Final tartaric acid concentration	4.98 g/L	4.98 g/L	4.98 g/L	4.98 g/L	4.98 g/L

*** The final concentration was calculated using: (the concentration after pH adjustment) x (8.33/10).**

3.4.2 GC-MS Analysis

Desorption and GC-MS analyses were carried out on a Shimadzu GCMS-QP2010 gas chromatograph–mass spectrometer equipped with a Combi-Pal auto sampler ready for automated SPME. Desorption of these volatiles occurred when the SPME fiber was inserted into the GC injection port for 5 minutes at 250 °C. GCMSsolutions version 2.72 was used as the data acquisition software. The chromatography was performed using an Rtx-Wax 60.0m x 0.25mm ID x 0.25µm film thickness (Polyethylene Glycol - Restek, Bellefonte, PA, USA) capillary GC column. Helium was used as the carrier gas with the GCMS set to a constant linear velocity of 29.2cm/sec. The injector was operated in split mode at a ratio of 10:1. The column oven was initially held at 45 °C for 8 minutes, then heated to 230 °C at 15 °C min-1 followed by a final ramp to 250 °C at 50 °C min-1 and held at this temperature for 10 minutes (See table 1 for elution times). Total run time was 30.73 minutes. The interface and MS source temperatures were set at 250 °C and 220 °C respectively. The MS was operated in electron impact mode (EI) at an ionization energy of 70eV. All analytes were analysed in full scan mode. Selected ions were used for the quantification of these analytes. Results were reported on an amount per volume basis i.e. µg/L.

Chapter 4

The Influence of Tannin and the Addition of Mucin on the Volatility and the Perceived Intensity of Ethyl Hexanoate in A Wine-Like Solution

4.1 Overview and Hypothesis

The overall goal of this study was to determine the influence of tannin and tannin-mucin interaction on the volatility and the perceived aroma intensity of ethyl hexanoate.

Research Hypotheses were:

1. Increasing tannin concentration decreases the volatility of ethyl hexanoate.
2. As the concentration of tannin increases, panelists may detect a decrease in the aroma intensity of ethyl hexanoate.
3. Adding salivary protein mucin may increase or decrease of the volatility of ethyl hexanoate. Direct binding of ethyl hexanoate with mucin would decrease the volatility, while tannin-mucin interaction could increase the volatility.
4. The change in the volatility of ethyl hexanoate resulting from the addition of mucin may result in the changes in the aroma intensity of ethyl hexanoate, which may be detected by the sensory panelists.

4.2 Materials and Methods

General Conditions for the Sensory Evaluation

Sensory analysis was carried out at Lincoln University, New Zealand, in the wine and food sensory room located in the Department of Wine, Food and Molecular Biosciences. There were 8 individual booths in the sensory room. The dimensions of the booths were 1.1 m (length)-0.8 (width)-2.7 m (height) (Falconer 2014). Each booth had side panels attached in order to prevent

interaction between assessors and its own build-in table top at a height of 0.72m and lighting source at a height of 2.0 m centrally above the booth (Falconer 2014). Temperature of the room was controlled at 20 ^{o}C. During the experiment, the temperature of the room remained constant. Tasting glasses used in this experiment were the black tasting glasses (ISO 3591:1977). Before each use, all wine glasses were placed upside down in customised racks and washed for two cycles in a commercial kitchen dishwasher (Starline GLV, New Zealand) using water at 90-95 °C without using any detergent. Each cycle took about 5 minutes. Once clean, the glasses were placed upright and left to air-dry. Petri dishes used as coverlids were cleaned for one cycle as above and after were removed from the dishwasher and left to air-dry.

Sensory Panelists

In total, 40 panelists (22 female and 18 male) participated in this experiment. 32 panelists (20 female and 12 male) participated in the sensory experiment on both days. They were volunteer students and staff at Lincoln University that were recruited via email. They understood that they were volunteering for this experiment. They were also aware that their personal information (e.g., age and experience in wine) was going to be collected, and might be used in data analysis and result interpretation. All panelists were over the age of 18 and had filled out the consent form requested by the Human Ethic Committee of Lincoln University. The study was approved by the Human Ethic Committee (LUHEC 2017-21). The age groups of the 40 panelists were: 18-24 years (23 panelists), 25-30 years (7 panelists), 31-44 years (9 panelists), and 45-60 years (1 panelist). In terms of their experience and knowledge of wine, four panelists claimed to be trained wine professionals and had previous wine judging and/or sensory panel experience and claimed to consume wine more than once a week. Five panelists had previous experiences in wine sensory analysis and considered themselves experienced tasters and claimed to consume wine more than once a week. Nine panelists considered themselves as regular wine consumers and their consumption of wine varied from 3 or 4 times a week to once a week. Eighteen panelists indicated that they were beginners at wine tasting and/or consumption and their consumption of wine varied from once a week to 3 to 11 times in the past year. Four panelists indicated that they were interested in wine, but interested in sensory experimentation, and had consumed wine 1 to 11 times in the past year.

Artificial Saliva & Wine-like solutions

Artificial saliva was prepared one day before the first sensory experiment and stored in the refrigerator at 4^{o}C. The methods of preparing the artificial saliva can be found in **Chapter 3**, section 3.3.2. Prior to use, artificial saliva was brought back to room temperature at 20^{o}C.

Wine-like solutions (i.e., control and T19.5, T6.48, T2.16, and T0.72) were prepared two days before the first sensory experiment and stored in the refrigerator at 4^{o}C. The methods of making and storing these solution can be found in **Chapter 3**, section 3.3.4. The night before the sensory experiment, wine-like solutions were taken out of the refrigerator and kept in the sensory preparation room at the controlled room temperature (i.e., set to 20^{o}C).

Test samples

The influence of tannin on the detection threshold estimates for ethyl hexanoate was examined on the first day. The test samples containing the control wine-like solution or the wine-like solution with tannins were prepared by adding 20 mL of the control or the tannin solutions into the black tasting wine glass (ISO 3591:1977). After that, 4 mL of distilled water was added into each glass and immediately covered with a petri dish. The addition of the distilled water was to dilute the concentrations of tannins, ethanol, and tartaric acid of the test samples to match the targeted concentrations. On day two, the influence of tannin and mucin interaction on the detection threshold estimates for ethyl hexanoate was examined. The test samples were prepared in the same way as before, but 4 mL of artificial saliva was added into each glass and covered with a petri dish. The final concentrations of tannin, ethanol, tartaric acid, and ethyl hexanoate in the test samples are shown in **Table 4.1 & Table 4.2 & Table 4.3**.

On both days of the experiment, preparation of the test samples was commenced at 8 AM and finished by 9 AM. The first session was at 9:30 AM, which had allowed samples to have 30 minutes for equilibration of the headspace before the first sensory session. Each sample was coded with a 3-digit number, as shown in **Figure 4.1**. The three samples for the same set were then placed onto a laminated paper with the set number written on the front (**Figure 4.2**). After each session, the old samples were discarded and new samples were prepared and allowed 30 minutes for equilibration of the headspace before the next session.

Table 4.1 Targeted and final tannin concentrations of control and tannin test samples for the sensory experiment.

Targeted tannin concentrations in the test samples	0 g/L (Control)	0.6 g/L	1.8 g/L	5.4 g/L	16.2 g/L
Tannin concentrations in wine-like solutions after pH adjustment	0 g/L	0. 71 g/L	2.15 g/L	6.46 g/L	19.46 g/L
Final tannin concentrations in the test samples after the addition of 4 mL RO water or artificial saliva	0 g/L	0.60 g/L	1.79 g/L	5.38 g/L	16.20 g/L

*** The final concentration was calculated using: (the concentration after pH adjustment) x (20 mL/24 mL).**

Table 4.2 Targeted and final ethyl hexanoate concentrations of control and tannin test samples for the sensory experiment**.**

Targeted ethyl hexanoate in the test samples	300 μg/L
Ethyl hexanoate concentration in the control and tannin solutions	360 μg/L
Final ethyl hexanoate concentration in the test samples after the addition of 4 mL RO water or artificial saliva	300 μg/L

*** The final concentration was calculated using: (the concentration after pH adjustment) x (20 mL/24 mL).**

Table 4.3 Targeted and final ethanol and tartaric acid concentrations of control and tannin test samples for the sensory experiment**.**

Targeted ethanol concentration in the test samples	12 %				
Ethanol concentrations in wine-like solutions after pH adjustment	T19.5	T6.48	T2.16	T0.72	Control
	14.36%	14.36%	14.36%	14.36%	14.38%
Final ethanol concentrations in the test samples after the addition of 4 mL RO water or artificial saliva	T16.2	T5.4	T1.8	T0.6	Control
	11.97%	11.97%	11.97%	11.97%	11.98%
Targeted tartaric acid concentration in the test samples	5 g/L				
Tartaric acid concentrations in wine-like solutions after pH adjustment	T19.5	T6.48	T2.16	T0.72	Control
	5.98 g/L	5.98 g/L	5.98 g/L	5.98 g/L	5.98 g/L
Final tannin concentrations in the test samples after the addition of 4 mL RO water or artificial saliva	T16.2	T5.4	T1.8	T0.6	Control
	4.98 g/L	4.98 g/L	4.98 g/L	4.98 g/L	4.98 g/L

*** The final concentration was calculated using: (the concentration after pH adjustment) x (20/24).**

Figure 4.1 Preparation of test samples for one test session. Picture was taken during the 30 minutes in which samples were equilibrating the headspace.

Experimental Design and Sensory Procedure

In this experiment, the influence of tannin was examined in an ascending order of the four tannin concentrations, following the method given by ASTM E679. At each tannin concentration step (i.e., each set), panelists received two control samples (C) and one wine-like solution with tannins (T), giving three possible presentation orders, i.e., CCT, CTC, TCC. At each set, the tasting orders were randomized and balanced across the 36 panelists (**Table A.1 and A.2, Appendix A**). The randomised order was generated using computer software Research Randomiser (Version 4.0). Also, the presentation order of samples was randomised within a panelist's series of the four triangle tests so that a panelist would encounter each of the three presentation orders at least once and no more than two times. These arrangements were based on the recommendations by Williams (1949), in order to balance for first order carry-over and position effects for each given odorant's concentration.

Five sensory sessions were carried out on each day (at 9:30 AM, 10:45 AM, 1:15 PM, 2:30 PM and 3:45 PM). Each session had 8 panelists (i.e., the maximum capacity of the sensory room), except the last session that had 4 panelists. On both days, panelists were seated randomly within each session and a number was assigned to each panelist. For example, if a panelist attended the first morning session, she/he would be getting a number between 1-8, while if a panelist attended the first afternoon session, she/he would be getting a number between 17-24. There were 36 numbers and none repeated. Each number was randomly assigned to a series of

triangle tests as shown in **Table A.1 and A.2**. On the second day of the study, the majority of the panelists due to personal reasons could not attend the session at the same time they did on the first day. In addition, 6 panelists that participated in the afternoon sessions on the first day attended the morning sessions on the second day.

Prior to each sensory session, a briefing/ warm -up session was carried out in the focus room, located next to the sensory room, where panelists met approximately 15 minutes before the formal sensory analysis. The pre-written script (**Appendix B**) used for the briefing session were based on the general instructions for the 3-AFC/triangle tests written by Lawless and Heymann (2010). During the warm-up procedure, the panelists were first instructed to smell two tannin samples with the lowest and the highest tannin concentrations and the control sample. They were then asked if they could detect any difference on the aroma intensity. Afterwards, the panelists were presented with a simple trial (i.e., two control samples and the tannin test sample with 5.4 g/L of tannins) and asked to practice and perform the sensory task in a correct manner. In order to be consistent with the instruction given by ASTM E679, the panelists were instructed to select the sample that was different from the other two. This type of instruction provided by ASTM E679 was not the conventional instruction for a 3-AFC test as discussed in **Chapter 3**, section 3.2.1, which was more likely to lead the panelists to use the "comparison of distances" strategy. In the present study, we chose to treat each sensory test (i.e., each set) as the triangle test and later process the sensory responses for significant detection based on the binomial distribution for the triangle test. The same way of dealing with this issue was described in Ross et al (2014) and Perry and Hayes (2016). Upon the completion of the warm-up session, all panelists had practiced the simple sensory trial and understood the task of the experiment.

The formal session was held in the sensory room. Each panelist was randomly seated in an individual booth equipped with a pen, general instructions, the sensory ballot (**Appendix B**) and a questionnaire (**Appendix B**). For the experiment, panelists were required to perform the four triangle tests in a clockwise sequence (i.e., staring from the first set ⟶the second set ⟶ the third set ⟶the fourth set). Each set had three samples, each coded with a three-digit number, and were placed onto a laminated paper with the set number on it (as seen in **Figure 4.2**). Panelists were required to smell the samples from left to right in order within each set. Re-smelling the samples was only allowed if it was for the entire set, which was done in the same order from left to right. Panelists were required to indicate the odd sample on the sensory ballot by circling the 3-digit number that corresponded to the chosen odd sample. For each triangle test, a choice must be made, even if the odd sample was not clearly detectable to the panelist. After giving a response in the first set, panelists were required to take one-minute break before starting the second set. To do this, panelists were asked to use their watch or the stopwatch on

their phone to time the one-minute break (see **Figure 4.3**). This was to minimise the carry-over and the sensory fatigue effects on panelists' judgement in the following set. The same procedure was applied through out the sensory experiment.

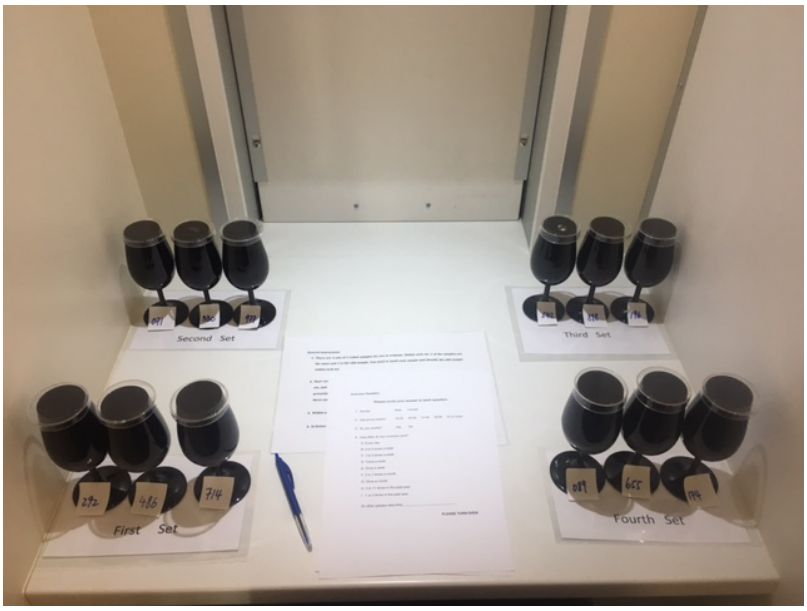

Figure 4.2 An example of the sensory test. The panelists were required to evaluate all four sets in a clockwise sequence (i.e., first set at bottom left, second set at top left, third set at top right and fourth set at bottom right).

Figure 4.3 A panelist using the stopwatch on his phone to time the one-minute break.

Instrumental Analysis

Test sample preparations and the procedures of instrumental analysis can be found in **Chapter 3**, section 3.3.5 and section 3.4.1 and 3.4.2. However, it was later found that 13μL of the ethyl hexanoate primary solution was added into the control and the tannin solutions, instead of 9 μL (i.e., the targeted volume to be added), giving 520 μg/L of ethyl hexanoate in the control and tannin solutions before the addition of the deionised water or the artificial saliva. After the addition of the deionised water or the artificial saliva, the final ethyl hexanoate concentration in each sample was finalised to be 437 μg/L that was verified by GC-MS.

Figure 4.4 The samples with the lowest (Left) and highest (Right) tannin concentrations prepared for HS-SPME-GC-MS analysis.

Data Analysis

The response for each triangle test was entered manually into an Excel spreadsheet (Microsoft Corporation 2010). Responses were coded as (-) where a panelist did not pick the tannin test sample or (+) where a panelist had chosen the tannin test sample as the odd sample, arranged in the order of the ascending tannin concentrations. The individual BET was taken as the geometric mean of the threshold estimates obtained from the four triangle tests. To calculate the individual BET, the Last Reversal rule was used. Therefore, the individual BET was calculated at

the concentrations where the panelist's judgment reversed from incorrect to correct for the last time. At times, where the threshold was above or below the studied concentration range, the threshold calculation was done by adding a hypothetical concentration step which was calculated by dividing or multiplying the lowest/highest tannin concentration step by 3 (i.e., the constant factor). For example, for panelists who did not pick out the tannin test sample at the highest tannin concentration, their individual BET was estimated as the geometric mean of the highest tannin concentration tested in this study and the next higher tannin concentration that would have been given if the series had been extended. For panelists that selected the tannin test sample at the lowest tannin concentration tested in this study, their individual BET was estimated as the geometric mean of the lowest tannin concentration tested in this study and the next lower tannin concentration. The frequencies of using the hypothetical concentrations were demonstrated in the next section. The group BET was calculated from the individual BETs using the method provided by ASTM E679.

Responses of each set were also analysed for significant detection based on the binomial distribution for the triangle test, as shown in **Appendix C** (Lawless and Heymann, 2010). Data were graphed in the Excel spreadsheet (Microsoft Corporation 2010) and the curve fitting and non-linear regression analysis were processed in XLSTAT (Addinsoft, Paris, France). To compare with the group BET results obtained using the method provided by ASTM E679, the group mean determined through the series of the four tests was achieved by extrapolating from the point at which the proportion of the panelists reached the criterion for significance (Lawless and Heymann, 2010). For the triangle test, using 36 or 32 panelists, to reach the 5% significance criterion, 50% of the panelists must perform correctly. However, in order to conclude a detection threshold, the required corrected proportion must be adjusted. This is due to the chance of guessing (i.e., the probability that a panelist should guess correctly, p=1/3) given in the triangle test (Lawless and Heymann, 2010). This was achieved by using the Abbott's formula: $P_{\text{required}} = (P_{\text{chance}} - P_{\text{correct}})/(1 - P_{\text{chance}})$, recommended by Lawless and Heymann (2010). In this formula, P_{required} is the required corrected proportion (i.e., used to conclude a detection threshold), P_{chance} is the chance of guessing, and P_{correct} is the threshold for a correct detection in a triangle test. In the present study, with n=36 or 32, the Prequired = $1/3 + 0.5(1-1/3)$ = 66.7% (Lawless and Heymann, 2010).

For comparing the individual BETs as well as the group BETs from before and after the addition of artificial saliva, data were excluded for panelists that failed to return for the second day. The data sets were first processed using the Shapiro-Wilk test (Shapiro and Wilk, 1965) and followed by the quantile-quantile plot in RStudio to check for the normality. Afterwards, the two data sets

were compared using the Wilcoxon signed-rank test (or commonly known as Wilcoxon rank sum test, Wilcoxon, 1945) (RStudio, Version 1.0.143, 2017). Wilcoxon signed-rank test was used instead of the t-test was because the two sets of individual BET values were not normally distributed. The theory of the Shapiro-Wilk test and Wilcoxon signed-rank test, and the R-scripts used to process the data and the results with significance defined as $P<0.05$ are demonstrated in **Appendix C**. The statistical analysis for comparing the headspace concentrations of ethyl hexanoate between the samples in each experiment was carried out using Tukey's HSD (honest significant difference) test. The theory of the Tukey's HSD is showing in **Appendix C**. For comparing the headspace concentrations before and after the addition of artificial saliva, the Shapiro-Wilk test and the Wilcoxon signed-rank test were then applied to the data.

4.3 Results

4.3.1 The Influence of Tannin on Aroma Perception and the Headspace Concentration of Ethyl Hexanoate

In the present study, the influence of tannin on the aroma intensity of ethyl hexanoate was defined at which 50% of the panelists could identify the tannin test sample as the odd sample. **Table C. 1** (**Appendix C**) provides the sensory responses from the 36 panelists in day 1. The number of panelists that chose the tannin test sample at each tannin concentration was counted manually in an Excel spreadsheet (Microsoft Corporation 2010). **Figure 4.5** demonstrates the proportion of panelists choosing the tannin test sample as the odd sample at each tannin concentration. At the two lower tannin concentrations (i.e., 0.6 and 1.8 g/L of tannins), there were only 13 panelists who chose the tannin test sample as the odd sample. While at the two higher tannin concentrations (i.e., 5.4 and 16.2 g/L of tannins), more than 50% of the panelists (i.e., 21 and 31 panelists, respectively) had chosen the tannin test sample as the odd sample. In addition, at 5.4 and 16 g/L of tannins, the sensory response had reached the 1% and 0.1% significance criterion, respectively, for the triangle test (reference **Table C.3**). It was clear that with the increase of the tannin concentration, the proportion of panelists choosing the tannin test sample as the odd sample was also increased.

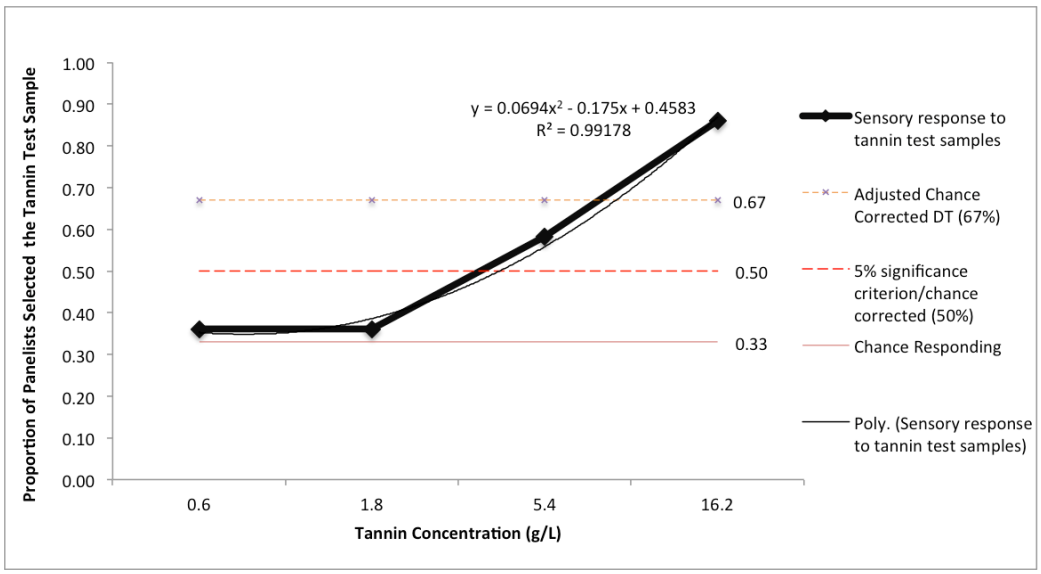

Figure 4.5 Proportion of panelists (n=36) selected the tannin test sample at each tannin concentration. The solid red line represents the chance responding (i.e., guessing probability), while the dashed red line indicates the 5% significance criterion using the binomial distribution for triangle test (i.e., 50% of the panelists). The dashed orange line is the corrected proportion of responses (66.7%) required to reach the 5% significance criterion for determining a detection threshold graphically.

Individual BETs (**Table C. 1**) were calculated using the method provided by ASTM E679, and the group BET was calculated as the geometric mean of the individual BETs. The method can be found in **Chapter 3**, section 3.2.2. Two panelists picked out the tannin test sample at all concentrations and five panelists did not pick out the tannin test sample at the highest tannin concentration. Therefore, the individual BET for these panelists was calculated by adding a hypothetical concentration step, as described in data analysis. In this study, Individual BET represented the best-estimated lowest tannin concentration at which the panelist should be able to identify a change of aroma intensity induced by tannins. **Figure 4.6** provides frequency distribution of individual BETs across the 36 panelists. In this experiment, the group BET was 4.68 g/L of tannins (as showing in **Table C.1, Appendix C**), which was the estimated lowest concentration at which 50% of the panelists should be able to identify the change of aroma intensity of ethyl hexanoate induced by tannins. In addition, the individual BET of 19 panelists was lower than the group BET, while the individual BET of the remaining 17 panelists was greater (more than two folds) than the group BET (**Figure 4.6)**. In addition, determined graphically, the detection threshold that was calculated based on the corrected proportion of responses (66.7%) required to reach the 5% significance criterion (i.e., the chance corrected DT in **Figure 4.5**) was extrapolated to be 3.41 g/L of tannins. The calculation was done in XLSTAT by applying 0.67 into the polynomial equation. Interestingly, the group BET value was greater than the detection threshold value determined through the series of the four sensory tests. Similar results with the group BET value (i.e., calculated via the method provided by ASTM E679) greater

than the detection threshold value determined from the 5% significance criterion over the panel were reported by Prescott et al (2005) and Ross et al (2014) in chemical threshold determination.

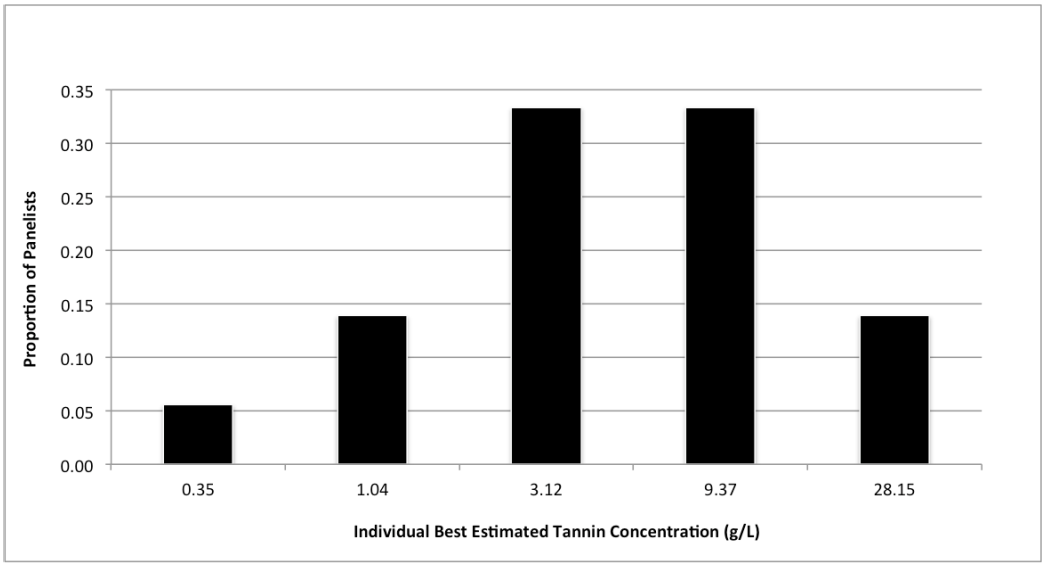

Figure 4.6 Frequency distribution of Individual BETs: the best estimated lowest tannin concentration at which the panelists should be able to identify the change of aroma intensity of ethyl hexanoate induced by tannins.

Figure 4.7 illustrates the headspace concentration of ethyl hexanoate in each sample measured using HS-SPME-GC-MS. It can be seen that with the increase of the tannin concentration, the headspace concentration of ethyl hexanoate was decreased (from 0 to 0.6 g/L of tannins) and then increased. This was not expected. This suggested that at lower concentrations, tannin decreased the volatility of ethyl hexanoate in the wine-like solution and that increasing tannin concentration increased the volatility of ethyl hexanoate from the solution. Tukey's multiple comparison test was used in RStudio to analyse the data (**Table 4.4**). In **Table 4.4**, the results show that the concentrations in the headspace of the samples were significantly different from each other. Another unexpected result was the headspace concentration of the control sample. The GC-MS results showed that the headspace concentrations of ethyl hexanoate in the control and the sample with the highest tannin concentration were very similar. However, according to the sensory data, significantly more panelists differentiated the tannin sample from the two control samples at the highest tannin concentration than at the lowest tannin concentration. Based on the sensory data, it would be expected that the difference in the headspace concentration of ethyl hexanoate between the control and the tannin sample with the highest concentration would be greater than that of the control sample and the sample with the lowest tannin concentration. Yet, in Figure 4.7, it can be seen that the concentration difference between the control sample and the sample with the lowest tannin concentration was greater.

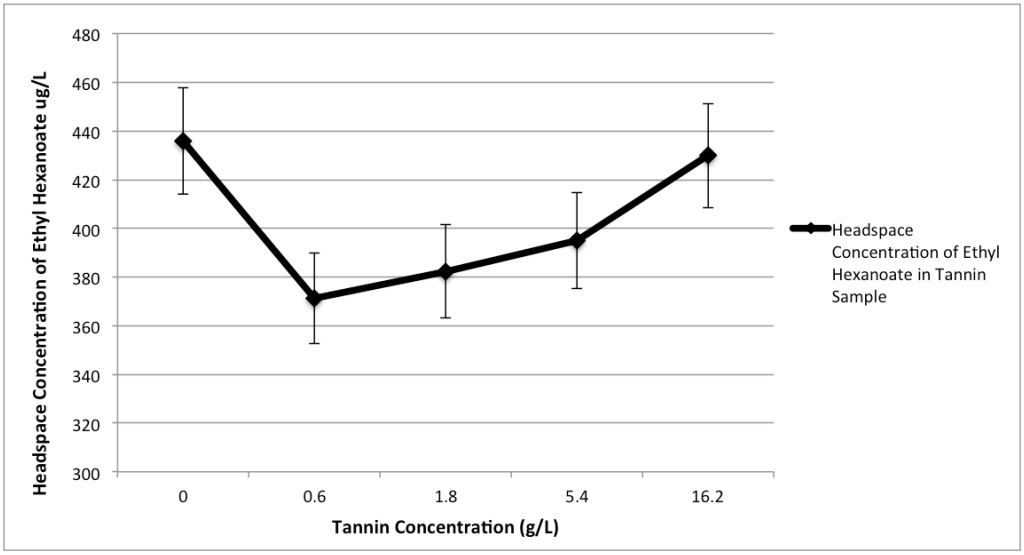

Figure 4.7 Headspace concentration of ethyl hexanoate in the samples measured using HS-SPME-GC-MS (Error bar with 5% significance criterion).

Table 4.4 Multiple comparison (Tukey test) of the average headspace concentration of ethyl hexanoate from tannin samples.

Tannin Concentration (g/L)	Ethyl hexanoate (μg/L)	Conf. Int.
16.2	429.9	A
5.4	395.05	B
1.8	382.3	C
0.6	371.25	D
Control	435.9	A

***R-scripts used for the statistical analysis and the full results can be found in Appendix C. Values described by the same letters do not differ significantly.**

4.3.2 The Influence of Mucin Addition

Sensory responses given by the panelists (n=36) in day 2 are showing in **Table C. 2** (**Appendix C**). In section 4.3.2, the results have included the responses and BET values of the panelists that did not participate in day 1. This was to treat the second experiment as an independent experiment first. In the next section 4.3.3, the responses and BET values were from the 32 panelists that

completed the sensory evaluations on both days. Once again, the number of panelists that selected the tannin test sample at each tannin concentration was counted in an Excel spreadsheet (Microsoft Corporation 2010). At 0.6 g/L of tannins, there were 7 panelists chose the tannin test sample as the odd sample, while at 1.8 g/L of tannins, there were 13 panelists who chose the tannin test sample as the odd sample. While, at the two higher tannin concentrations (i.e., 5.4 and 16.2 g/L of tannins), more than 50% of the panelists (i.e., 25 and 29 panelists, respectively) had selected the tannin test sample as the odd sample. This time at these two concentrations, the sensory responses had reached the 0.1% significance criterion for the triangle test (as seen in the reference **Table C.3**).

Individual BETs (**Table C.2**) were calculated using the method provided by ASTM E679. Two panelists picked out the tannin test sample at all concentrations and seven panelists did not pick out the tannin test sample at the highest tannin concentration studied in this experiment. As mentioned before, the individual BET for these panelists was calculated by adding a hypothetical concentration step. The proportion of panelists who chose the tannin test sample at each tannin concentration and the frequency distribution of individual BETs across the panelists are shown in **Figure 4.8** and **4.9,** respectively. The group BET was calculated as the geometric mean of the individual BETs. In this experiment, the group BET was 3.89 g/L of tannins (as showing in **Table C.2, Appendix C**), which was the estimated lowest concentration at which 50% of the panelists should be able to identify the change of aroma intensity when tannins were added. Overall, there were 23 panelists that had individual BET values lower than the group BET, while 13 panelist had BET values higher (more than 2-fold) than the group BET. In addition, the group BET calculated using this method was found to be greater than the group mean (i.e., 3.05 g/L of tannins) determined graphically from the corrected proportion of responses (66.7%) required to reach the 5% significance criterion (i.e., the chance corrected DT in **Figure 4.8**).

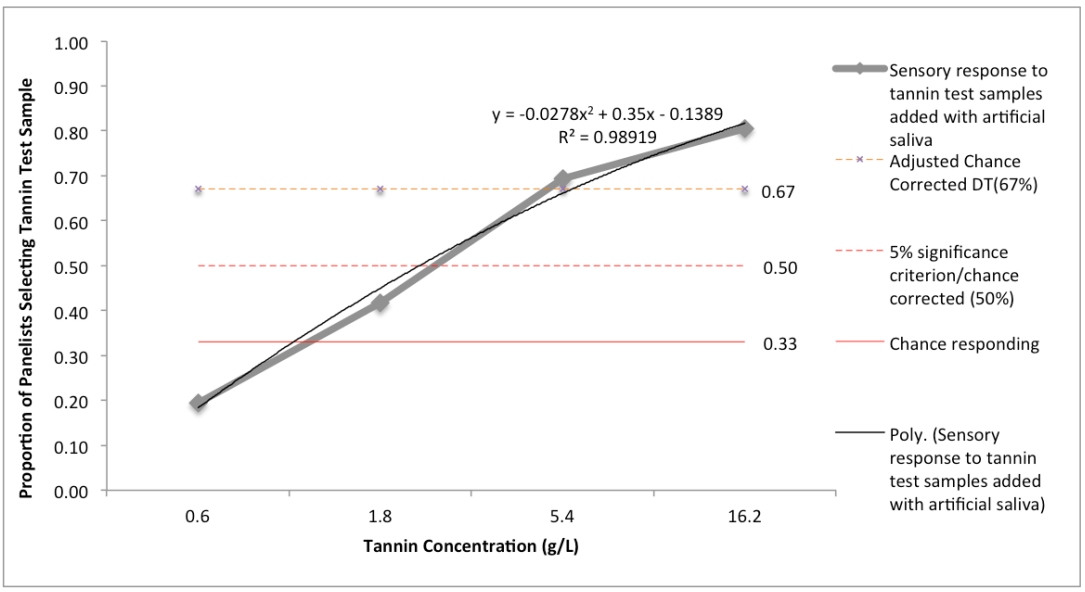

Figure 4.8 When mucin was added: the proportion of panelists choosing the tannin test sample at each tannin concentration. The solid red line represents the chance responding (i.e., guessing probability), while the dashed red line indicates the 5% significance criterion using the binomial distribution for triangle tests (n=36). The dashed orange line is the corrected proportion of responses (66.7%) required to reach the 5% significance criterion for determining a detection threshold graphically.

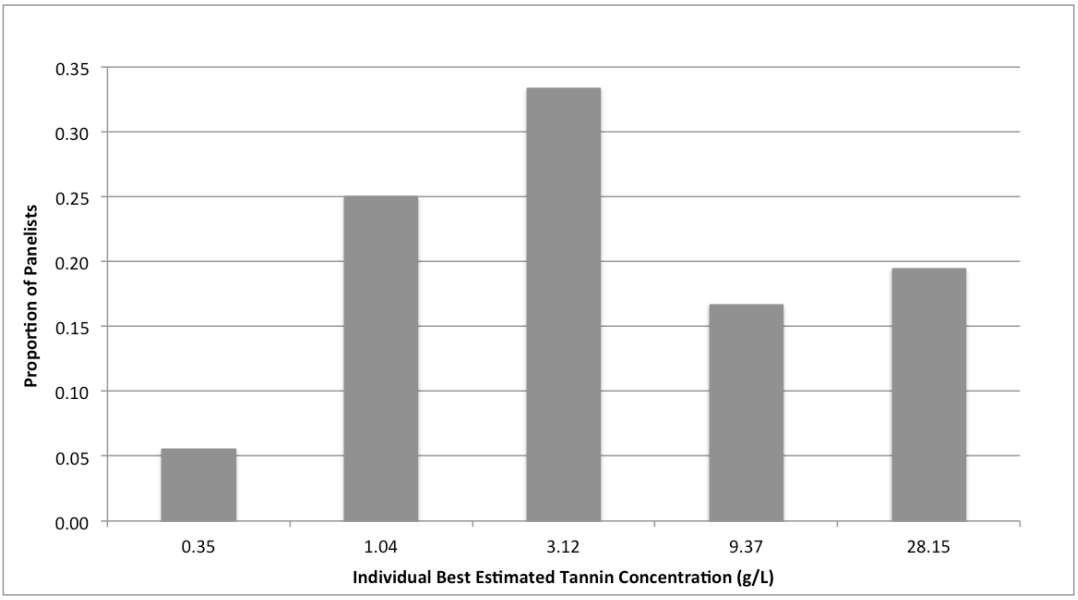

Figure 4.9 Frequency distribution of individual BETs, when mucin was added.

Figure 4.10 illustrates the headspace concentration of ethyl hexanoate from tannin samples containing mucin measured using HS-SPME-GC-MS. It can be seen that the ethyl hexanoate headspace concentration found in the sample with 16.2 g/L of tannins was close to the concentration found in the sample with 1.8 g/L of tannins. Data were compared using the Tukey's multiple comparison test in RStudio. The results (**Table 4.5**) showed that a significant difference was found between the headspace concentrations of the tannin samples with 16.2 g/L and 5.4 g/L of tannins and then between samples with 5.4 g/L and 1.8 g/L of tannins. Also, it can be seen that the headspace concentrations of ethyl hexanoate in the lowest and the highest tannin samples were not significantly different. Therefore, it would be expected that the proportions of correct responses from the sensory experiment at the two concentrations would be similar. Yet, the sensory study showed that significantly more panelists gave the correct response at the highest tannin concentration step. This finding is discussed below.

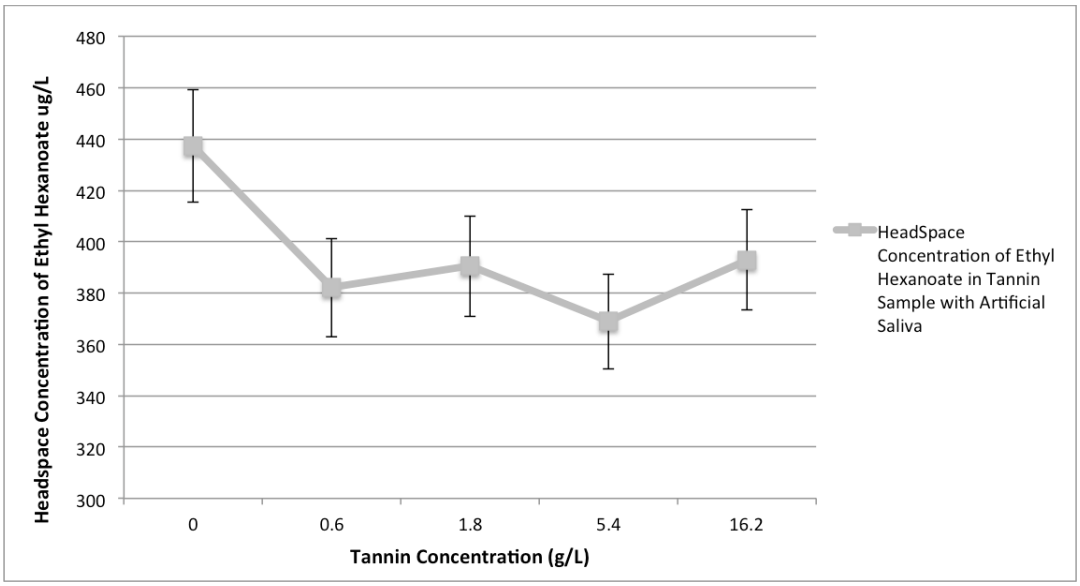

Figure 4.10 Headspace concentration of ethyl hexanoate from tannin samples added with artificial saliva measured using HS-SPME-GC-MS (Error bar with 5% significant criterion).

Table 4.5 Multiple comparison (Tukey test) of the average headspace concentration of ethyl hexanoate from tannin samples with added artificial saliva.

Tannin Concentration (g/L)	Ethyl hexanoate (μg/L)	Conf. Int.
16.2	392.95	A
5.4	368.9	B
1.8	390.45	AC
0.6	382.1	ABC
Control	437.3	D

***R-scripts used for the statistical analysis and the full results can be found in Appendix C, values described by the same letters do not differ significantly ($p<0.05$).**

4.3.3 Individual BETs (n=32) and the Group BETs from Before and After the Addition of Artificial Saliva

As shown in **Table 4.6**, individual BETs with and without the addition of mucin were matched for the same panelist. Individual panelists were coded with different letters. Because the majority of the panelists could not attend the session on the second day at the same time they did on the first day, they were assigned with different numbers and therefore giving the randomisation similar over the two-day studies. For the panelists that did attend the same sensory session on both days, as they were seated randomly, some of them still had different numbers (as seen in **Table C.4, Appendix C**). As mentioned before, each number was randomly assigned to a series of the four triangle tests with randomized presentation orders.

Comparing the results from the two experiments (**Table 4.6**), it showed that with the addition of mucin, the group BET was slightly lowered (i.e., from the 4.27 to the 3.72 g/L of tannins). In terms of the individual BETs, the results showed that the individual BETs of 15 panelists were lowered, 9 panelists were increased and 8 panelists had no change. The frequency distribution of individual best estimated tannin concentrations before and after the addition of artificial saliva can be found in **Figure 4.11**. Within the 15 panelists, 6 panelists' BET was decreased from 9.37 to 3.12 g/L of tannins (their sensory response (+) using the last reversal rule was changed from the 16.2 to the 5.4 g/L tannin concentration); 2 panelists' BET was decreased from 9.37 to 1.04 g/L of tannins (sensory response (+) changed from the 16.2 to the 3.12 g/L tannin concentration); 4 panelists' BET was decreased from 3.12 to 1.04 g/L of tannins (sensory response (+) changed from the 5.4 to the 1.8 g/L tannin concentration); the remaining 3 panelists had their BETs that decreased from 9.37 to 0.35 g/L, 3.12 to 0.35 g/L, and 28.15 to 9.37

g/L of tannins, respectively, with their sensory response (+) changed from the 16.2 to the 0.2 g/L (the lower hypothetical concentration step), the 5.4 to the 0.2 g/L tannin concentration, and the 48.6 (the higher hypothetical concentration step) to 16.2 g/L of tannin concentration, respectively. For the 9 panelists that had increased individual BETs, 2 panelists' BET was increased from 1.04 to 28.15 g/L of tannins (sensory response (+) changed from the 1.8 to the 48.6 g/L tannin concentration); 1 panelist's BET was increased from 3.12 to 28.15 g/L (sensory response (+) changed from the 5.4 to the 48.6 g/L concentration); 1 panelist's BET was increased from 1.04 to 3.12 and another panelist's BET was increased from 1.04 to 9.37 g/L (sensory response (+) changed from the 1.8 to the 5.4 g/L tannin concentration and the 1.8 to 16.2 g/L tannin concentration, respectively); 2 panelists' BET was increased from 0.35 to 1.04 g/L and from 0.35 to 3.12 g/L, respectively (sensory response (+) changed from the 0.2 to the 1.8 g/L tannin concentration and 0.2 to 5.4 g/L tannin concentration, respectively); and 2 panelists' BET was increased from 3.12 to 9.37 g/L (sensory response (+) changed from the 5.4 to the 16.2 g/L tannin concentration).

Table 4.6 Summary of the individual best-estimate thresholds (BETs) in $\log_{10}$ units, the group BETs and standard errors that were obtained from the two experiments with 32 panelists**.**

Panelists	Day 1 Best-Estimate threshold (BET)		Day 2 Best-Estimate threshold (BET)		Changes of BET	
	Value	Log 10 of Value	Value	Log 10 of Value		
A	3.12	0.49	28.15	1.45	∧	
B	9.37	0.97	3.12	0.49	∨	
C	1.04	0.02	28.15	1.45	∧	
D	0.35	-0.46	1.04	0.02	∧	
E	3.12	0.49	3.12	0.49	=	
F	9.37	0.97	1.04	0.02	∨	
G	9.37	0.97	3.12	0.49	∨	
H	3.12	0.49	1.04	0.02	∨	
I	1.04	0.02	28.15	1.45	∧	
J	0.35	-0.46	3.12	0.49	∧	
K	28.15	1.45	28.15	1.45	=	
L	3.12	0.49	1.04	0.02	∨	
M	1.04	0.02	1.04	0.02	=	
N	28.15	1.45	28.15	1.45	=	
O	28.15	1.45	9.37	0.97	∨	
P	3.12	0.49	3.12	0.49	=	
Q	3.12	0.49	9.37	0.97	∧	
R	9.37	0.97	9.37	0.97	=	
S	9.37	0.97	3.12	0.49	∨	
T	3.12	0.49	1.04	0.02	∨	
U	9.37	0.97	3.12	0.49	∨	
V	9.37	0.97	1.04	0.02	∨	
W	9.37	0.97	0.35	-0.46	∨	
X	9.37	0.97	3.12	0.49	∨	
Y	1.04	0.02	9.37	0.97	∧	
Z	1.04	0.02	3.12	0.49	∧	
AA	3.12	0.49	0.35	-0.46	∨	
AB	3.12	0.49	3.12	0.49	=	
AC	3.12	0.49	9.37	0.97	∧	
AD	28.15	1.45	28.15	1.45	=	
AE	9.37	0.97	3.12	0.49	∨	
AF	3.12	0.49	1.04	0.02	∨	
Summary	∑log 10 -->	20.07	∑log 10 -->	18.18	∧	9
	Average	0.63	Average	0.57	∨	15
	BET	4.27	BET	3.72	=	8
	Std	0.52	Std	0.57	N.A	4

***The data of panelists that did not participate in both days were excluded. The symbol '∧' represented the panelist's individual BET was increased; '∨' represented the panelist's individual BET was decreased; '=' represented the panelist's individual BET did not change.**

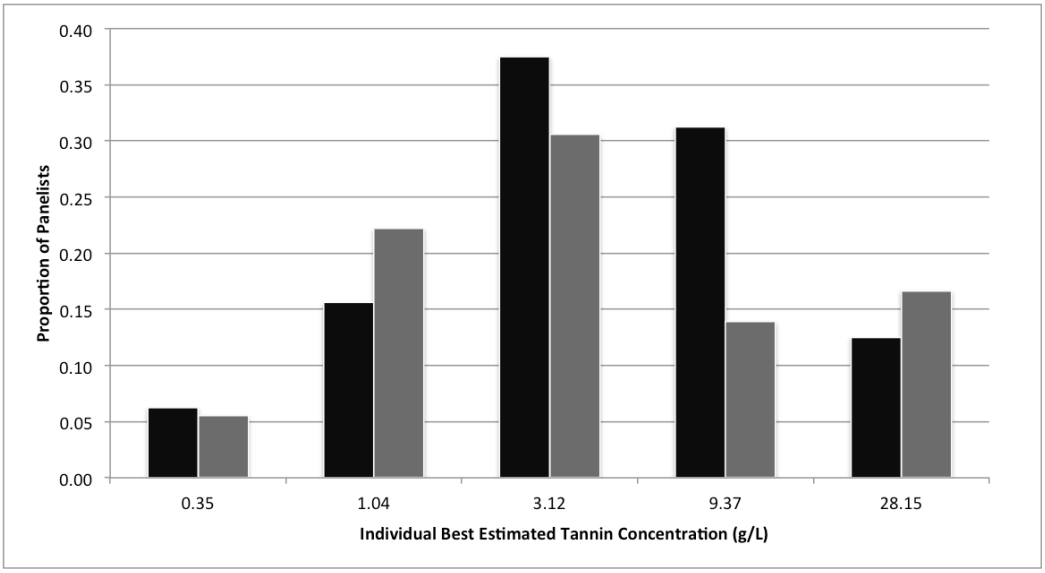

Figure 4.11 Frequency distribution of individual best estimated tannin concentrations found in the two sensory experiments (n=32). Responses and BET values of the panelists that did not participate in both days were excluded.

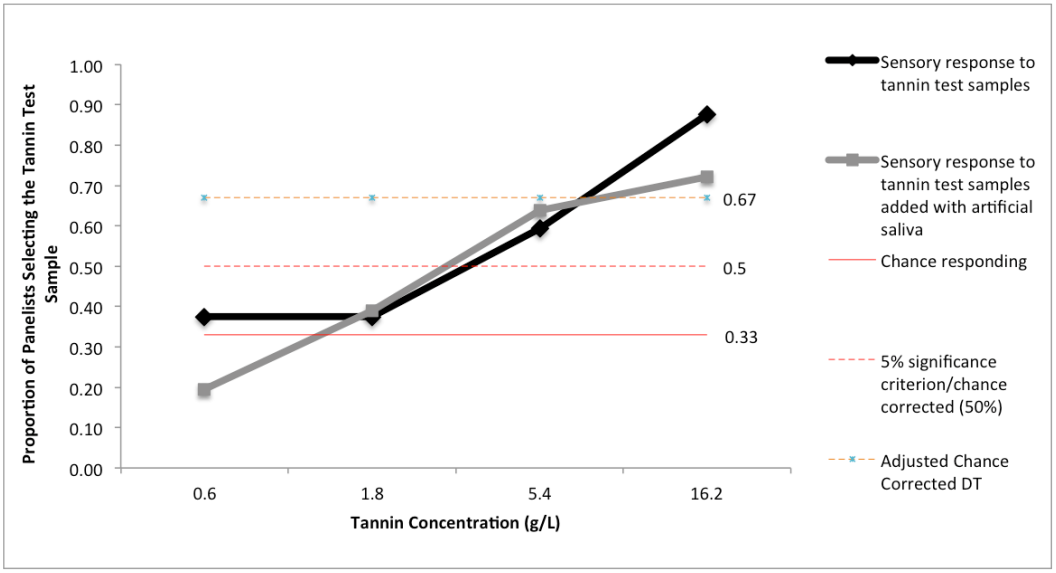

Figure 4.12 Comparison of sensory responses from Day 1 (with tannins only) and Day 2 (tannins added with artificial saliva). Responses of panelists that did not participate in both days were excluded. The solid red line represents the chance responding (i.e., guessing probability), while the dashed red line indicates the 5% significance criterion (i.e., 50% of the panelists) using the binomial distribution for triangle tests (n=32). The dashed orange line is the corrected proportion of responses (66.7%) required to reach the 5% significance criterion for determining a detection threshold graphically.

In addition, using the sensory responses from the 32 panelists who attended both sensory experiments, the detection threshold calculated graphically (**Figure 4.12**) at the corrected response threshold (i.e., 67%) was recalculated for each experiment (XLSTAT). It was found that the detection threshold in the first experiment was changed from 3.41 g/L (n=36) to 3.37 g/L (n=32), while in the second experiment the detection threshold was changed from 3.05 g/L (n=36) to 3.45 g/L (n=32). Both DT values were still lower than the group BETs.

Besides these observations, the data obtained from the two experiments were also analysed using a paired difference test. Prior to this, Shapiro-Wilk test for normality (RStudio) was used to determine if the two sets of individual BET values were normally distributed and was followed by applying the Q-Q plot to confirm the results. The theory of the Shapiro-Wilk test can be found in section C.5 (**Appendix C**). The null-hypothesis of Shapiro-Wilk test is always that the population is normally distributed. Therefore, if the p-value is less than 0.05, then the null hypothesis is rejected and it means the data tested are not from a normally distributed population. The results, as seen in section C.6 (**Appendix C**), showed that neither of the two sets of data was normally distributed (i.e., $P < 0.05$ for both data sets), which were confirmed in the Q-Q plots. Therefore, F test and paired t-test could not be used. Instead, the Wilcoxon signed-rank test was used. The theory of the Wilcoxon signed-rank test can be found in section C.5. R-scripts used for the statistical analyses and the outcomes can be found in section C.6. The null hypothesis of the test in the present study was that the two population means were equal with difference between the pairs (n=32) of individual BETs (i.e., before and after the addition of artificial saliva) following a symmetric distribution around zero. The results showed the *P*-value was greater than 0.05. Therefore, this demonstrated that the addition of mucin did not induce any significant shift on the estimated lowest tannin concentration value at which 50% of the panelists should be able to identify the change of aroma intensity of ethyl hexanoate induced by tannins.

4.3.4 Aroma Release of Ethyl Hexanoate from Tannin Samples

In terms of the influence of mucin on the headspace concentrations of ethyl hexanoate from the tannin samples (Figure 4.13), it can be seen that mucin caused some variation in the volatility of ethyl hexanoate in the tannin samples. Before the addition of mucin, increasing tannin concentration increased the volatility of ethyl hexanoate, but after the addition of mucin, the same trend was not found. At higher tannin concentrations, when mucin was added, the volatility of ethyl hexanoate was lower than that without mucin. While, at the highest tannin concentration, without mucin, the volatility of ethyl hexanoate in the sample was almost the

same as found in the control sample, but after the addition of mucin, it was reduced by 37 µg/L (i.e., 8.6% lower) (**Table 4.7**). The changes of the headspace concentration of ethyl hexanoate from before and after the addition of artificial saliva were calculated (i.e., giving the absolute values) and analysed in RStudio using the Tukey test, as shown in Table 4.6. Rscripts used for data analysis and the outcomes can be found in Appendix C. The results showed that the change of the headspace concentration of ethyl hexanoate in the tannin sample with 16.2 g/L of tannins was significant different when compared to the tannin samples with 0.6 and 1.8 g/L of tannins, respectively.

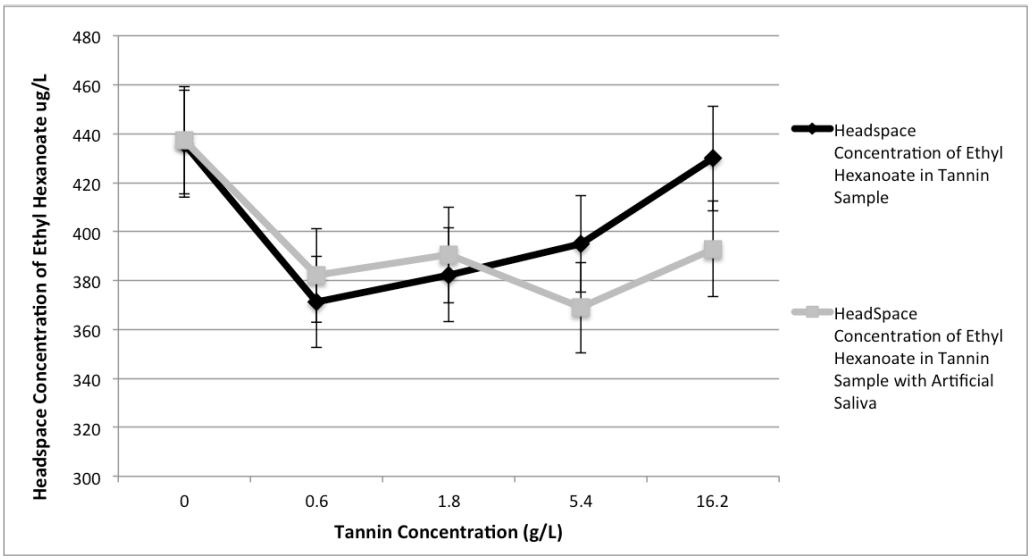

Figure 4.13 Headspace concentration of ethyl hexanoate before and after the addition of artificial saliva measured using HS-SPME-GC-MS (Error bar with 5% significant criterion).

Table 4.7 Multiple comparison (Tukey test) of the changes of the averaged headspace concentration of ethyl hexanoate from tannin samples before and after the addition of artificial saliva.

Tannin Concentration (g/L)	Ethyl hexanoate (μg/L) Δ=|after − before| the addition of artificial saliva	Conf. Int.
16.2	36.95	**A**
5.4	26.15	**AB**
1.8	8.15	**BC**
0.6	10.85	**BC**

***The values are the absolute values and values described by the same letters do not differ significantly.**

4.4 Discussion

According to the results from the instrumental analysis, it was found that with the increase of tannin concentration, the aroma release of ethyl hexanoate was also increased. Similar results were also found in Munoz-Gonzalez et al (2013) and Munoz-Gonzalez (2014). In Munoz-Gonzalez (2014), white wine (with 270 mg/L of total polyphenols) and red wine (1648 mg/L of total polyphenols) were both added with 890 μg/L of ethyl hexanoate. The GC-MS results showed that in the headspace of the white wine, there was 179.9 μg/L of ethyl hexanoate, while in the red wine headspace, there was 201.4 μg/L of ethyl hexanoate. Gonzalez et al (2013) also reported that volatility of ethyl hexanoate was found to be higher in red wine than in white wine. However, Aronson and Ebeler (2004) found that with the addition of tannin (i.e., 1.5 g/L) decreased the concentration of ethyl hexanoate in the headspace (i.e., reduced by 52 μg/L). In addition, Mitropoulou et al (2011) demonstrated that increasing tannin concentration from 1 g/L to 10 g/L decreased the concentration of ethyl hexanoate in the headspace. Gonzalez et al (2013) and Munoz-Gonzalez (2014) both used complex reconstituted wine matrices, whereas Aronson and Ebeler (2004) and Mitropoulou et al (2011) both used simple wine-like solutions that were similar to the wine-like solution used in the present study. Dufour and Bayonove (1999) suggested that the decrease in solubility of hydrophobic compounds at lower tannin concentrations (i.e., studied in the range 5-20 g/L) could be associated with the prevention of hydrophobic binding due to structural reasons (e.g., lower catechin content), and for the volatility of hydrophobic compounds to be decreased, the matrix would require higher levels of seed tannins (i.e., catechin). It has been found that grape skin tannins contain long polymeric

chains (with degrees of polymerization: DP ranging from 3 to 83) and are composed of procyanidins and prodelphinidins (McRae and Kennedy 2011). While the grape seed tannins contain lower average degree of polymerisation (i.e., between DP 2 to17) than skin tannins and consist of mainly catechin and epicatechin subunits (McRae and Kennedy 2011). Several studies had then used the 4:1 seed to skin tannin ratio to study influence of tannin on the aroma release. For the present study, the 4:1 seed to skin tannin ratio was used. However in the present study, ethyl hexanoate was the only aroma compound in the matrix, whereas in the studies described above multiple aroma compounds were added to the matrices. With a number of aroma compounds present at the same time, interactions between aroma compounds as well as between aroma compounds and the matrix would be expected. It has been demonstrated that interactions between aroma compounds could also result in the decrease of the volatility of some aroma compounds (Pineau et al 2007, Ferreira 2010). In the present study, at the two lower tannin concentrations, the reduced volatility of ethyl hexanoate might have resulted from the interaction between ethyl hexanoate and catechin from seed tannins. Jung and Ebeler (2003) demonstrated that catechin had shown to interact selectively with aroma compounds, in which the volatility of ethyl hexanoate was reduced by 10-20%. The sample at the highest tannin concentration step had shown similiar binding ability as the control sample to ethyl hexanoate, in which the concentration of ethyl hexanoate in the headspace was almost the same as the concentration found in the headspace of the control solution. This might be due to tannin self-association, which decreased the number of reactions sites to bind with the aroma compound. The mechanism of self-association was first described in Asen et al (1972). Catechin self-association has been demonstrated in Tobiason et al (1999).

Based on the results from the instrumental analysis, it was expected that at the highest tannin concentration, the detection rate (or the correct response) for the tannin sample (i.e., without the addition of artificial saliva) would not reach the 5% significant criterion for the triangle test, as statistically there should be no significant difference in the aroma intensity between the control and the tannin sample. However, this contradicted the results from the sensory experiment. For the sensory experiments, the data showed that with increasing tannin concentration, the proportion of panelists making the correct response (i.e., selecting the tannin sample a the odd sample from each set) also increased. This would suggest that at the two higher tannin concentrations, the difference between the headspace concentrations of ethyl hexanoate in the control sample and the tannin sample should be greater than when compared to the difference between the control and the tannin sample at the lower concentrations. During the sample preparation for instrumental analysis and before the SPME sampling, the samples were agitated from 10 min in a sealed vial with a 1:1 liquid to headspace volume ratio,

whereas for the sensory experiment, the samples were pipetted into tasting glasses (with a 1:7.6 liquid to headspace volume ratio) and were only agitated after the sample preparation by the experimenter and during the sensory analysis by the panelists. Therefore, in this case, it would be expected that the actual headspace concentration of ethyl hexanoate might not be the same in these two vessels. Rabe et al (2004) investigated the best optimal headspace to sample volume ratio (i.e., 1:1, 3:1, and 1:3) for aroma analysis and reported that the use of 1:1 liquid to headspace volume ratio had led to a significant increase of the headspace aroma concentration. In addition, instrumental analysis measured the total aroma release of ethyl hexanoate from the solutions during the 10-minute agitation and 2-minute extraction, because ethyl hexanoate was released and then absorbed onto the fiber. Therefore, a constant extraction would alter the liquid-headspace equilibrium of the aroma compound to release the remaining ethyl hexanoate from the solution. But for the sensory experiment, firstly, the liquid to headspace volume ratio was not the same as for the instrumental analysis, therefore the aroma release might be different; secondly, a panelist would sniff the aroma in the headspace but would not continuously 'extracting' the aroma from the vessel for two minutes. Secondly, the first sniff would extract the aroma compound from the headspace, then might alter the liquid-headspace equilibrium of the aroma compound, which might induce the further release of the aroma compound from the solution into the headspace. At equilibrium, the relationship between the concentration of aroma compound in the liquid phase and in the gaseous phase can be expressed by Henry's law. This law describes that the mass of gas dissolved in a certain volume of solvent is directly proportional to the partial pressure of the gas that is in equilibrium with the solution (i.e., $K_H=P_v/C$, K_H is the constant, P_v is partial pressure of the gas and C is the concentration in the solvent). In the present study, static headspace analysis extracted the aroma compound of the equilibrium headspace surrounding the sample solution, however, this procedure does not simulate the actual sensory perception. Thus, the amounts determined do not necessarily represent the aroma compound and the quantity available for perception during sensory analysis.

Instrumental analysis also showed that the effect of adding mucin at higher tannin concentrations reduced the volatility of ethyl hexanoate to be approximately equal to that for the lower tannin concentrations. This reduction in volatility might have resulted from the interaction between the aroma compound, tannins and mucin. Because, without adding mucin, the aroma release of the highest tannin sample was close to that for the control sample and later in the control sample added with artificial saliva, the volatility of ethyl hexanoate did not change (i.e., the interaction was not simply between the aroma compound and mucin). Therefore, this reduced headspace concentration of ethyl hexanoate was most likely induced by

the influence of tannin-mucin complexes on the aroma compound as demonstrated by Munoz-Gonzalez et al (2014). It has been demonstrated that tannins bind to salivary proteins in three distinct stages (McRae and Kennedy 2011). The initial interactions between tannin and salivary proteins involve both hydrophobic interactions and hydrogen bonding and result in the formation of protein-tannin complexes (McRae and Kennedy 2011). During the second stage, the formation of protein aggregates with bound tannins occur, which is via self-association, resulting in the cross-links between the previously formed protein-tannin complexes (McRae and Kennedy 2011). The final stage of tannin-protein interaction occurs when the protein aggregates eventually merge and produce colloidal particles, which then leads to the precipitation of protein-tannin complexes (McRae and Kennedy 2011). Furthermore, in vitro and in vivo studies of the influence of saliva and/or artificial saliva on the aroma release from liquids had reported that the saliva flow rate, air-flow rate and the liquid-headspace surface area (i.e., 1:1) also had significant effects on the aroma release from the solution (Buettner et al 2002, Rabe et al 2004, Munoz-Gonzales et al 2014). Therefore, in this experiment, for the instrumental analysis the samples that were sealed immediately after the addition of artificial saliva and agitated for 10 minutes in a sealed vial with 1:1 headspace-liquid ratio would be expected to show more accurate changes in the aroma release induced by the addition of mucin. This needs to be further investigated.

4.5 Conclusion

1. With increasing tannin concentration, panelists detected a change in perception of the aroma compound, which was reflected in the increased number of panelists correctly selecting the tannin sample. This was not affected by the addition of mucin.

2. The perception changes detected by the panelists were not likely to have resulted from the changes in the volatility of ethyl hexanoate, as firstly, largest reduction in volatility was found at lowest tannin concentration, and secondly, adding mucin did not significantly affect the perception but did affect the volatility of ethyl hexanoate, especially at the highest tannin concentration.

3. Without the addition of mucin, the reduction in volatility found at lower tannin concentrations was likely due to direct interaction between tannins and the aroma compound. At higher tannin concentrations, it was speculated that the increased volatility of ethyl hexanoate was possibly due to tannin self-association that formed self-association complexes that were not able to interact with the aroma compound. However, this was not further investigated in the present study, therefore, requiring future experiments to validate this speculation.

4. With the addition of mucin, at higher tannin concentrations, the influence of tannins on the volatility of ethyl hexanoate was suspected to be disrupted by the formation of tannin-mucin complexes, which resulted in the decreased volatility of ethyl hexanoate in the tannin samples.

Chapter 5

Summary and Future Work

Summary of the study

The first obstacle encountered in this study was the concern that grape extracted tannins could contribute some unexpected aromas. In the wine aroma studies by Aronson and Ebeler (2004), Carvalho et al (2006), Munoz-Gonzalez et al (2013), Mitropoulou et al (2011), similar commercial tannins were use to reconstitute the wine matrix that was then used to prepare the wine-like solutions. These studies used various concentrations of tannins, from 0.5 g/L to up to 10 g/L of tannins, yet, none of these studies, except Aronson and Ebeler (2004) had discussed the possibility of the grape extracted tannins retaining some aromas. In the study by Aronson and Ebeler (2004), the authors described that the tannin solution (i.e., 16 g/L) used for reconstituting the wine matrix was flushed with nitrogen at room temperature for 30 minutes to remove trace aromas. The same method was also attempted in the present study but failed as the resultant tannin solution (at 16 g/L and 32 g/L respectively) was not devoid of trace aromas. Another attempt on dearomatising the tannin solution was carried out using a rotary evaporator. This method was described in Carvalho et al (2006) for grape seed tannin isolation. However, attempted at room temperature, the resulting tannin solution still displayed the trace aromas. The method of dearomatisation used in the present study, which was using the 4 g/L of LiChrolut EN resins, was shown to be a more successful method in terms of removing the unexpected aromas from grape extracted tannins. Future studies that require the reconstitution of wine matrix using commercial tannins should not neglect the trace aromas in the commercial tannins and when necessary use appropriate dearomatisation method to remove the trace aromas.

One other concern was the sensory instruction given by ASTM E679. In ASTM E679, the method declares the use of a series of 3-AFC tests. But it is not clear why the method has given a sensory instruction that is similar to the sensory instruction of the triangle test. As already discussed, the 3-AFC test has a higher power than the triangle test, that is because the 3-AFC test has a more optimal cognitive strategy, which is resulted from the use of a more clear sensory instruction. Therefore, to conclude a detection for a stimulus, the required proportions of correct responses using the 3-AFC and the triangle tests are different (Bi 2006, *pp*.184). Although, the method provided by ASTM E679 for calculating the individual BETs as well as the group BET does not require using the proportion of correct responses from each concentration step, for studies where the proportions of correct responses between each concentration step are compared or

used to determine the detection threshold graphically, the required correct responses for each concentration step must be known. In the present study the group BETs calculated using the method provided by ASTM E679 were higher than the detection thresholds determined graphically using the proportions of correct responses from each concentration step. Ross et al (2014) and Perry and Hayes (2016) also reported the variations between the group BET calculated using the method provided by ASTM E679 and the graphically determined detection threshold. Future studies may need to investigate the cause of this variation as it has been reported but never looked into.

For the present study, without knowing the change in the volatility of ethyl hexanoate (i.e., increase or decrease) resulting from tannins or the tannin-protein interaction, the sensory question (i.e., "which sample is the odd one") had to be unspecific. If it had been known that increasing tannin concentration induced an increase in the volatility of ethyl hexanoate, then in the sensory experiment, the panelists could have been asked, "which sample had higher aroma intensity of ethyl hexanoate." Therefore, for example, if most panelists could not correctly choose the tannin sample at lower concentrations, but with the increasing tannin concentration more than 50% of the panelists then chose the tannin sample as the odd sample with higher aroma intensity, then we might conclude that increasing tannin concentration resulted in an increased intensity of the aroma of ethyl hexanoate that could be detected via the orthonasal pathway. Without a specific question, this restricted the conclusion that could be made from the sensory data.

Future work

Future work needs to investigate what caused the reduced binding ability of tannins (e.g., some form of self-association) to the aroma compound at the highest tannin concentration step. In addition, the present study investigated the influence of tannin and tannin-mucin on the perceived aroma intensity of ethyl hexanoate at 300 μg/L, but did not determine the influence of tannin or tannin-mucin on the perception threshold and odor activity value (OAV) of ethyl hexanoate. The perception threshold of ethyl hexanoate is at 14 μg/L (Escudero et al 2007). Currently, the perception thresholds of many volatile compounds were determined in a water solution, which might not be the same if determined in a wine-like solution or a wine matrix (Francis and Newton 2005, Ferreira et al 2016). The OAV, often called aroma or odour units, is a useful measure to assess the relative importance of individual aroma compounds present in a specific matrix (Francis and Newton 2005). In wine studies, the OAV of an aroma is calculated by dividing the concentration of that aroma compound in a wine by the perception threshold

concentration for that aroma compound. As mentioned earlier, the perception thresholds of many volatile compounds were determined in a water solution, thus, the OAV values calculated using these perception thresholds might not represent their true values in wine.

A similar sensory experiment can be applied to measure the perception threshold of ethyl hexanoate as well as many other wine aroma compounds in a wine-like solution. This can be determined by using the same sensory methods as in the present study with the concentrations of ethyl hexanoate from 14 μg/L (i.e., the lowest concentration) to 300 μg/L (i.e., the highest concentration). At each concentration step, there will be one sample containing ethyl hexanoate and two blank samples containing no ethyl hexanoate. The perception threshold of ethyl hexanoate in a wine-like solution will be calculated as the group BET value. After obtaining the perception threshold, the OAV of ethyl hexanoate can be calculated as well, which then can be compared to the OAV values obtained in the previous studies. In addition, with the updated perception threshold, the influence of tannin or tannin-mucin interaction on the perception threshold can be determined. This can be carried out following the same experimental methods as described in the present study.

Appendix A

Experimental Design & Sample Presentations

A.1 Day 1: Sample Presentations of the Four Triangle tests Across the Panelists (n=36).

Table A.1 Day 1: Sample Presentations Encountered by the Panelists, with the presentation order of samples randomised within a panelist's series of the four triangle tests**.**

n=36 Panelists	First Set (IV)			Second Set (III)			Third Set (II)			Fourth Set ( )		
1	C	T0.6	C	T1.8	C	C	C	T5.4	C	C	C	T16.2
2	C	C	T0.6	C	T1.8	C	C	C	T5.4	T16.2	C	C
3	T0.6	C	C	C	C	T1.8	T5.4	C	C	C	T16.2	C
4	C	C	T0.6	C	T1.8	C	C	T5.4	C	T16.2	C	C
5	C	T0.6	C	T1.8	C	C	T5.4	C	C	C	C	T16.2
6	T0.6	C	C	C	C	T1.8	C	C	T5.4	C	T16.2	C
7	C	T0.6	C	T1.8	C	C	C	T5.4	C	C	C	T16.2
8	C	C	T0.6	C	T1.8	C	C	C	T5.4	T16.2	C	C
9	T0.6	C	C	C	C	T1.8	T5.4	C	C	C	T16.2	C
10	T0.6	C	C	C	C	T1.8	T5.4	C	C	C	T16.2	C
11	C	T0.6	C	T1.8	C	C	C	C	T5.4	c	C	T16.2
12	C	C	T0.6	C	T1.8	C	C	T5.4	C	T16.2	C	C
13	C	C	T0.6	C	T1.8	C	T5.4	C	C	T16.2	C	C
14	C	T0.6	C	T1.8	C	C	C	T5.4	C	C	C	T16.2
15	T0.6	C	C	C	C	T1.8	C	C	T5.4	C	T16.2	C
16	C	T0.6	C	T1.8	C	C	T5.4	C	C	C	C	T16.2
17	T0.6	C	C	C	C	T1.8	C	T5.4	C	C	T16.2	C
18	C	C	T0.6	C	T1.8	C	C	C	T5.4	T16.2	C	C
19	C	T0.6	C	C	C	T1.8	C	C	T8	T16.2	C	C
20	T0.6	C	C	C	T1.8	C	C	T5.4	C	C	C	T16.2
21	C	C	T0.6	T1.8	C	C	T5.4	C	C	C	T16.2	C
22	C	C	T0.6	C	T1.8	C	C	C	T5.4	T16.2	C	C
23	T0.6	C	C	C	C	T1.8	C	T5.4	C	C	T16.2	C
24	C	T0.6	C	T1.8	C	C	T5.4	C	C	C	C	T16.2
25	C	T0.6	C	C	C	T1.8	C	T5.4	C	T16.2	C	C
26	T0.6	C	C	T1.8	C	C	C	C	T5.4	C	T16.2	C
27	C	C	T0.6	C	T1.8	C	T5.4	C	C	C	C	T16.2
28	T0.6	C	C	T1.8	C	C	C	T5.4	C	C	C	T16.2
29	C	C	T0.6	C	T1.8	C	T5.4	C	C	C	T16.2	C
30	C	T0.6	C	C	C	T1.8	C	C	T5.4	T16.2	C	C
31	C	C	T0.6	C	C	T1.8	C	T5.4	C	T16.2	C	C
32	C	T0.6	C	C	T1.8	C	T5.4	C	C	C	C	T16.2
33	T0.6	C	C	T1.8	C	C	C	C	T5.4	C	T16.2	C
34	C	C	T0.6	T1.8	C	C	T5.4	C	C	C	T16.2	C
35	C	T0.6	C	C	T1.8	C	C	C	T5.4	T16.2	C	C
36	T0.6	C	C	C	C	T1.8	C	T5.4	C	C	C	T16.2

Table A.2 An Example of the 3-digit Codes Used in the Day 1 Experiment**.**

Coded Test Samples for Examining Tannin's Influence												
n=36 Assessor	First Set (IV)			Second Set (III)			Third Set (II)			Fourth Set ( )		
1	486	292	714	977	071	330	818	542	196	089	174	655
2	486	714	292	071	977	330	818	196	542	655	089	174
3	292	486	714	071	330	977	542	818	196	089	655	174
4	486	714	292	071	977	330	818	542	196	655	089	174
5	486	292	714	977	071	330	542	818	196	089	174	655
6	292	486	714	071	330	977	818	196	542	089	655	174
7	486	292	714	977	071	330	818	542	196	089	174	655
8	486	714	292	071	977	330	818	196	542	655	089	174
9	292	486	714	071	330	977	542	818	196	089	655	174
10	292	486	714	071	330	977	542	818	196	089	655	174
11	486	292	714	977	071	330	818	196	542	089	174	655
12	486	714	292	071	977	330	818	542	196	655	89	174
13	486	714	292	071	977	330	542	818	196	655	089	174
14	486	292	714	977	071	330	818	542	196	089	174	655
15	292	486	714	071	330	977	818	196	542	089	655	174
16	486	292	714	977	071	330	542	818	196	089	174	655
17	292	486	714	071	330	977	818	542	196	089	655	174
18	486	714	292	071	977	330	818	196	542	655	089	174
19	486	292	714	071	330	977	818	196	542	655	089	174
20	292	486	714	071	977	330	818	542	196	089	174	655
21	486	714	292	977	071	330	542	818	196	089	655	174
22	486	714	292	071	977	330	818	196	542	655	089	174
23	292	486	714	071	330	977	818	542	196	089	655	174
24	486	292	714	977	071	330	542	818	196	089	174	655
25	486	292	714	071	330	977	818	542	196	655	089	174
26	292	486	714	977	071	330	818	196	542	089	655	174
27	486	714	292	071	977	330	542	818	196	089	174	655
28	292	486	714	977	071	330	818	542	196	089	174	655
29	486	714	292	071	977	330	542	818	196	089	655	174
30	486	292	714	071	330	977	818	196	542	655	089	174
31	486	714	292	071	330	977	818	542	196	655	089	174
32	486	292	714	071	977	330	542	818	196	089	174	655
33	292	486	714	977	071	330	818	196	542	089	655	174
34	486	714	292	977	071	330	542	818	196	089	655	174
35	486	292	714	071	977	330	818	196	542	655	089	174
36	292	486	714	071	330	977	818	542	196	089	174	655

A.2 Day 2: Sample Presentations of the Four Triangle tests Across the Panelists (n=36).

Table A.3 Day 2: Sample Presentations Encountered by the Panelists, with the presentation order of samples randomised within a panelist's series of the four triangle tests**.**

n=36 Panelists	First Set (VIII)			Second Set (VII)			Third Set (VI)			Fourth Set (V)		
1	C	C	T0.6	C	T1.8	C	T5.4	C	C	C	C	T16.2
2	C	T0.6	C	T1.8	C	C	C	C	T5.4	T16.2	C	C
3	T0.6	C	C	C	C	T1.8	C	T5.4	C	C	T16.2	C
4	C	C	T0.6	C	T1.8	C	C	T5.4	C	T16.2	C	C
5	C	T0.6	C	T1.8	C	C	T5.4	C	C	C	C	T16.2
6	T0.6	C	C	C	C	T1.8	C	C	T5.4	C	T16.2	C
7	C	T0.6	C	C	T1.8	C	T5.4	C	C	C	C	T16.2
8	T0.6	C	C	C	C	T1.8	C	C	T5.4	C	T16.2	C
9	C	C	T0.6	T1.8	C	C	C	T5.4	C	T16.2	C	C
10	C	T0.6	C	T1.8	C	C	C	C	T5.4	C	T16.2	C
11	C	C	T0.6	C	C	T1.8	C	T5.4	C	T16.2	C	C
12	T0.6	C	C	C	T1.8	C	T5.4	C	C	C	C	T16.2
13	C	C	T0.6	C	T1.8	C	C	C	T5.4	T16.2	C	C
14	C	T0.6	C	C	C	T1.8	T5.4	C	C	C	T16.2	C
15	T0.6	C	C	T1.8	C	C	C	T5.4	C	C	C	T16.2
16	C	T0.6	C	T1.8	C	C	C	C	T5.4	T16.2	C	C
17	T0.6	C	C	C	T1.8	C	C	T5.4	C	C	C	T16.2
18	C	C	T0.6	C	C	T1.8	T5.4	C	C	C	T16.2	C
19	T0.6	C	C	C	T1.8	C	C	C	T5.4	T16.2	C	C
20	C	C	T0.6	T1.8	C	C	C	T5.4	C	C	CW	T16.2
21	C	T0.6	C	C	C	T1.8	T5.4	C	C	C	T16.2	C
22	C	C	T0.6	T1.8	C	C	C	C	T5.4	C	T16.2	C
23	T0.6	C	C	C	C	T1.8	C	T5.4	C	T16.2	C	C
24	C	T0.6	C	C	T1.8	C	T5.4	C	C	C	C	T16.2
25	C	C	T0.6	T1.8	C	C	C	T5.4	C	C	T16.2	C
26	C	T0.6	C	C	C	T1.8	C	C	T5.4	T16.2	C	C
27	T0.6	C	C	C	T1.8	C	T5.4	C	C	C	C	T16.2
28	C	T0.6	C	T1.8	C	C	C	C	T5.4	T16.2	C	C
29	T0.6	C	C	C	T1.8	C	C	T5.4	C	C	C	T16.2
30	C	C	T0.6	C	C	T1.8	T5.4	C	C	C	T16.	C
31	T0.6	C	C	T1.8	C	C	C	T5.4	C	C	C	T16.2
32	C	T0.6	C	C	C	T1.8	T5.4	C	C	C	T16.2	C
33	C	C	T0.6	C	T1.8	C	C	C	T5.4	T16.2	C	C
34	C	C	T0.6	C	T1.8	C	T5.4	C	C	C	C	T16.2
35	C	T0.6	C	C	C	T1.8	C	T5.4	C	T16.2	C	C
36	T0.6	C	C	T1.8	C	C	C	C	T5.4	C	T16.2	C

Table A.4 An Example of the 3-digit Codes Used in the Day 2 Experiment**.**

Coded Test Samples for 3-AFC Test for Examining Tannin-Salivary Protein Interaction												
n=36	First Set (VIII)			Second Set (VII)			Third Set (VI)			Fourth Set (V)		
Assessor												
1	948	651	768	491	877	265	333	237	559	536	937	052
2	948	768	651	877	491	265	237	559	333	052	536	937
3	768	948	651	491	265	877	237	333	559	536	052	937
4	948	651	768	491	877	265	237	333	559	052	536	937
5	948	768	651	877	491	265	333	237	559	536	937	052
6	768	948	651	491	265	877	237	559	333	536	052	937
7	948	768	651	491	877	265	333	237	559	536	937	052
8	768	948	651	491	265	877	237	559	333	536	052	937
9	948	651	768	877	491	265	237	333	559	052	536	937
10	948	768	651	877	491	265	237	559	333	536	052	937
11	948	651	768	491	265	877	237	333	559	052	536	937
12	768	948	651	491	877	265	333	237	559	536	937	052
13	948	651	768	491	877	265	237	559	333	052	536	937
14	948	768	651	491	265	877	333	559	237	536	052	937
15	768	948	651	877	491	265	237	333	559	536	937	052
16	948	768	651	877	491	265	237	333	559	052	536	937
17	768	948	651	491	877	265	237	559	333	536	937	052
18	948	651	768	491	265	877	333	237	559	536	052	937
19	768	948	651	491	877	265	237	559	333	052	536	937
20	948	651	768	877	491	265	237	333	559	536	927	052
21	948	768	651	491	265	877	333	237	559	536	052	937
22	948	651	768	877	491	265	237	559	333	536	052	937
23	768	948	651	491	265	877	237	333	559	052	536	937
24	948	768	651	491	877	265	333	237	559	536	937	052
25	948	651	768	877	491	265	237	333	559	536	052	937
26	948	768	651	491	265	877	237	559	333	052	536	937
27	768	948	651	491	877	265	333	237	559	536	937	052
28	948	768	651	877	491	265	237	559	333	052	536	937
29	768	948	651	491	877	265	237	333	559	536	937	052
30	948	651	768	491	265	877	333	237	559	536	052	937
31	768	948	651	877	491	265	237	333	559	536	937	052
32	948	768	651	491	265	877	333	237	559	536	052	937
33	948	651	768	491	877	265	237	559	333	052	536	937
34	948	651	768	491	877	265	333	237	559	536	937	052
35	948	768	651	491	265	877	237	333	559	536	052	937
36	768	948	651	877	491	265	237	559	333	052	536	937

Appendix B

Pre-written Scripts Used for Sensory Briefing Session

B.1 Scripts for the Briefing Session

Steps:

1. *Hand out questionnaire*

2. *Statement of the objective of the study*

This experiment is to evaluate the intensity of wine aroma.

For today and tomorrow's experiment, there are 4 sets of 3 coded samples with 2 samples are the same and 1 sample is the odd sample. You are required to assess all 4 sets of 3 samples by smelling only, so tasting is not allowed.

3. *Demonstration of the test*

1. Each of you will be sitting in an individual booth in the sensory room next door. This is to help you get focused on the experiment. Once the analysis starts, there is no talking with each other or any kind of interaction with each other.

2. During the course of this experiment, you are only allowed to use the direct orthonasal olfaction, i.e., to use your nose to evaluate samples. Tasting/consumption is not allowed.

3. There are 4 sets of 3 samples all served in black glasses. In total, 12 glasses of samples. The set up of each set is exact the same like what we have here in the focus room. Each glass is covered with a plastic petri dish cap. When you ready to smell the sample, you bring the glass to your nose and take off the cap. Smell it just like you would normally smell a glass of wine. Afterwards, you put the cap back on and put the glass back to the same place. And you do the same on the second and third sample.

4. In this experiment, sensory evaluation must start from the first set then to the second set, then the third set and lastly to the fourth set. While within each set, analysis must always start from left to right.

5. Within each set, you may re-smell the samples, but only if it is for the entire set, and you must re-smell the samples from left to right.

6. Eventually you must make a choice and indicate the sample that is the odd one to you. Even if you are not sure, you must make a choice. Once you have made up your choice, you need to circle the sample's 3-digit code on the sensory ballot.

7. You cannot start the second set, if you have not finish the first set.

8. Show panelists how to fill out the ballot.

9. Every time you finish a set, fill out the result sheet. Afterwards, before you start the next set, you need to take a one-minute break. This is for minimizing the sensory adaptation and fatigue effects. You can time the break either using your watch or the stopwatch on your phone.

10. Once the one-minute is up, you start the next set, again from left to right. Repeat the sensory process that you have done on the samples in the first set. Again, you may re-smell the samples, but only if it is for the entire set, and you must re-smell the samples from left to right. When you have made the decision, circle the odd sample's 3-digit number on the ballot and answer the question.

11. Once you finish the second set, fill out the ballot. Afterwards, again take a one-minute break before you get onto the third set.

12. The same procedures apply to the third and fourth set. Any question?

13. Once you finish all three sets you can leave all the paper works on the desk and leave the sensory room.

14. Ask panelists to practice the sensory procedure using a simple trial. Make sure they have practiced the procedures and understood the tasks.

B.2 General Instruction (Approved: LUHEC 2017-21)

Instructions for Sensory Evaluation of Wine Aroma

1. There are 4 sets of 3 coded samples for you to evaluate. Within each set, two of the samples are the same and one is different. You need to smell each sample and identify the odd sample within each set.

2. Start sensory evaluation with the first set, then move to the second set, then to the third set, and lastly to the fourth set.

3. Within each set, smell each of the 3 samples in the sequence presented, from left to right. You may re-smell the samples only if it is for the entire set and please follow the sequence from left to right.

4. Within each set, you must indicate which sample is the odd one by circling its 3-digit number on the sensory ballot.

5. In between sets, make sure you take a one-minute break.

B.3 Questionnaire Given to the Panelists (Approved: LUHEC 2017-21)

Assessor Number: 1

Please circle your answer to each question

1. Gender Male Female
2. Age group (years): 18-24 25-30 31-44 45-60 61 or more
3. Do you smoke? Yes No
4. How often do you consume wine?
   - A. Every day
   - B. 5 to 6 times a week
   - C. 3 to 4 times a week
   - D. Twice a week
   - E. Once a week
   - F. 2 to 3 times a month
   - G. Once a month
   - H. 3 to 11 times in the past year
   - I. 1 or 2 times in the past year

   Or other (please describe)_____

PLEASE TURN OVER

5. Do you have any wine evaluation experience (e.g. sensory panelist, wine judge)?

   A. Yes please comment_____

   B. No

6. Do you consider yourself_____

   A. a wine expert.

   B. a trained and knowledgeable wine professional.

   C. a passionate wine lover.

   D. a regular consumer

   E. a beginner.

   F. you are not into wine.

Or other (please describe)_____

B.4 Sensory Ballot (Approved: LUHEC 2017-21)

Assessor No._____1_____

Within each set, you must indicate the odd sample by circulating the sample's 3-digit code.

1. **Start the first set from left to right. Within the first set, which sample is the odd one?**

   486 292 714

   (**After you finish the first set, take a one-minute break**)

2. **Start the second from left to right. Within the second set, which sample is the odd one?**

   977 071 330

   (**After you finish the second set, take a one-minute break**)

Within each set, you must indicate the odd sample by circulating the sample's 3-digit code.

3. **Start the third set from left to right. Within the third set, which sample is the odd one?**

   818 542 196

   (**After you finish the third set, take a one-minute break**)

4. **Start the fourth set from left to right. Within the fourth set, which sample is the odd one?**

   089 174 655

Thank you for your participation!

Appendix C

Sensory Responses, Individual and Group BETs, Statistical Analysis Using R-scripts in RStudio

C.1 Summary of the sensory responses from Day 1

Table C.1 Detection threshold estimate for ethyl hexanoate under the influence of tannins, summary of the individual best-estimate thresholds (BETs) in $\log_{10}$ units and standard errors that were obtained from the 36 individual BETs**.**

Panelists	Day 1, Sensory Judgements at Five Tannin Concentrations (g/L)				Best-Estimate threshold (BET)	
	0.6	1.8	5.4	16.2	Value	Log 10 of Value
1	+	-	+	+	3.12	0.49
2	-	+	-	+	9.37	0.97
3	-	+	+	+	1.04	0.02
4	+	+	+	+	0.35	-0.46
5	+	-	+	+	3.12	0.49
6	-	+	-	+	9.37	0.97
7	+	-	-	+	9.37	0.97
8	+	-	-	+	9.37	0.97
9	-	-	+	+	3.12	0.49
10	-	+	+	+	1.04	0.02
11	+	-	-	+	9.37	0.97
12	+	+	+	+	0.35	-0.46
13	+	-	+	-	28.15	1.45
14	-	-	+	+	3.12	0.49
15	-	+	+	+	1.04	0.02
16	+	+	-	-	28.15	1.45
17	-	-	-	-	28.15	1.45
18	+	-	+	+	3.12	0.49
19	-	-	+	+	3.12	0.49
20	-	-	-	+	9.37	0.97
21	-	+	-	+	9.37	0.97
22	-	-	+	+	3.12	0.49
23	-	-	-	+	9.37	0.97
24	-	-	+	+	3.12	0.49
25	+	-	-	+	9.37	0.97
26	-	-	-	+	9.37	0.97
27	-	-	-	+	9.37	0.97
28	-	+	+	+	1.04	0.02
29	-	+	+	+	1.04	0.02
30	+	-	+	+	3.12	0.49
31	+	-	+	+	3.12	0.49
32	-	-	+	+	3.12	0.49
33	-	+	-	-	28.15	1.45
34	-	+	-	+	9.37	0.97
35	-	-	+	-	28.15	1.45
36	-	-	+	+	3.12	0.49
Group BET geometric mean					∑log 10 -->	23.95
					Average	0.67
					BET	4.68
					Std	0.51
Note: '+' represents a panelist selected the tannin test sample; ' -' represents a panelist did not select the tannin test sample.						

***Panelists assigned with the number 6, 11, 24 and 35 failed to return for the second day of the experiment.**

C.2 Summary of the sensory responses from Day 2

Table C.2 Detection threshold estimate for ethyl hexanoate under the influence of tannin-salivary protein interaction, summary of the individual best-estimate thresholds (BETs) in $\log_{10}$ units and standard errors that were obtained from the 36 individual BETs**.**

Panelists	Day 2, Sensory Judgements at Five Tannin Concentrations (g/L) + Artificial Saliva				Best-Estimate threshold (BET)	
	0.6	1.8	5.4	16.2	Value	Log 10 of Value
1	-	-	-	+	9.37	0.97
2	+	-	+	+	3.12	0.49
3	-	+	+	+	1.04	0.02
4	-	+	+	+	1.04	0.02
5	-	+	+	+	1.04	0.02
6	-	-	-	-	28.15	1.45
7	-	-	-	+	9.37	0.97
8	-	-	-	-	28.15	1.45
9	-	-	-	-	28.15	1.45
10	-	+	-	-	28.15	1.45
11	-	-	+	+	3.12	0.49
12	-	+	-	-	28.15	1.45
13	+	+	+	+	0.35	-0.46
14	-	-	+	+	3.12	0.49
15	-	-	+	+	3.12	0.49
16	-	+	+	+	1.04	0.02
17	-	-	+	+	3.12	0.49
18	-	+	-	+	9.37	0.97
19	-	-	+	+	3.12	0.49
20	+	-	+	+	3.12	0.49
21	-	-	+	+	3.12	0.49
22	-	-	-	+	9.37	0.97
23	-	+	+	+	1.04	0.02
24	-	-	-	+	9.37	0.97
25	+	+	+	+	0.35	-0.46
26	-	-	+	+	3.12	0.49
27	+	-	+	-	28.15	1.45
28	+	-	-	+	9.37	0.97
29	-	-	+	+	3.12	0.49
30	-	+	+	+	1.04	0.02
31	-	+	+	+	1.04	0.02
32	+	+	+	-	28.15	1.45
33	-	-	+	+	3.12	0.49
34	-	+	+	+	1.04	0.02
35	-	+	+	+	1.04	0.02
36	-	-	+	+	3.12	0.49
Group BET geometric mean					∑log 10 -->	21.11
					Average	0.59
					BET	3.89
					Std	0.57
Note: '+' represents a panelist selected the tannin test sample;' -' represents a panelist did not select the tannin test sample.						

*** For the second day of the experiment, panelists assigned with the number 3, 22, 29 and 32 did not participate in the sensory experiment in day 1.**

C.3 Reference Table of the Critical Values for the Triangle Test

Table C.3 The number of assessors in a triangle test required to give correct judgements, at three different significant level (Lawless and Heymann, 2010).

Number of Assessors	Significance Level		
	5%	1%	0.1%
7	5	6	7
8	6	7	8
9	6	7	8
10	7	8	9
11	7	8	10
12	8	9	10
13	8	9	11
14	9	10	11
15	9	10	12
16	9	11	12
17	10	11	13
18	10	12	13
19	11	12	14
20	11	13	14
21	12	13	15
22	12	14	15
23	12	14	16
24	13	15	16
25	13	15	17
26	14	15	17
27	14	16	18
28	15	16	18
29	15	17	19
30	15	17	19
31	16	18	20
32	16	18	20
33	17	18	21
34	17	19	21
35	17	19	22
36	18	20	22
37	18	20	22
38	19	21	23
39	19	21	23
40	19	21	24
41	20	22	24
42	20	22	25
43	20	23	25
44	21	23	26
45	21	24	26
50	23	26	28

C.4 Sensory Reuslts from 32 Panelists with Individual BETs Matched to the Panelists

Table C.4 Summary of the individual best-estimate thresholds (BETs) in $\log_{10}$ units, the geometric means obtained from the two experiments with 32 panelists.

Panelists	No. in Day 1	No. in Day 2	Day 1 Best-Estimate threshold (BET)		Day 2 Best-Estimate threshold (BET)		Changes of BET	
			Value	Log 10 of Value	Value	Log 10 of Value		
A	1	10	3.12	0.49	28.15	1.45	Λ	
B	2	15	9.37	0.97	3.12	0.49	V	
C	3	8	1.04	0.02	28.15	1.45	Λ	
D	4	34	0.35	-0.46	1.04	0.02	=	
E	5	17	3.12	0.49	3.12	0.49	=	
F	7	5	9.37	0.97	1.04	0.02	V	
G	8	19	9.37	0.97	3.12	0.49	V	
H	9	16	3.12	0.49	1.04	0.02	V	
I	10	27	1.04	0.02	28.15	1.45	Λ	
J	12	2	0.35	-0.46	3.12	0.49	Λ	
K	13	9	28.15	1.45	28.15	1.45	=	
L	14	35	3.12	0.49	1.04	0.02	V	
M	15	31	1.04	0.02	1.04	0.02	=	
N	16	12	28.15	1.45	28.15	1.45	=	
O	17	22	28.15	1.45	9.37	0.97	V	
P	18	21	3.12	0.49	3.12	0.49	=	
Q	19	18	3.12	0.49	9.37	0.97	Λ	
R	20	28	9.37	0.97	9.37	0.97	=	
S	21	11	9.37	0.97	3.12	0.49	V	
T	22	4	3.12	0.49	1.04	0.02	V	
U	23	20	9.37	0.97	3.12	0.49	V	
V	25	23	9.37	0.97	1.04	0.02	V	
W	26	25	9.37	0.97	0.35	-0.46	V	
X	27	36	9.37	0.97	3.12	0.49	V	
Y	28	7	1.04	0.02	9.37	0.97	Λ	
Z	29	29	1.04	0.02	3.12	0.49	Λ	
AA	30	13	3.12	0.49	0.35	-0.46	V	
AB	31	14	3.12	0.49	3.12	0.49	=	
AC	32	1	3.12	0.49	9.37	0.97	Λ	
AD	33	6	28.15	1.45	28.15	1.45	=	
AE	34	33	9.37	0.97	3.12	0.49	V	
AE	34	33	9.37	0.97	3.12	0.49	V	
AF	36	30	3.12	0.49	1.04	0.02	V	
Group Geometric Mean Summary			∑log 10 -->	20.07	∑log 10 -->	18.18	Λ	8
			Average	0.63	Average	0.57	V	15
			BET	4.27	BET	3.72	=	9
			Std	0.52	Std	0.57	N.A	4

***The data of panelists that did not participate in both days were excluded.**

C.5 Statistical Analysis: the Shapiro-Wilk test, the Wilcoxon rank sum test, and the Tukey HSD test.

Shapiro-Wilk test

The null hypothesis of the test is that the population is normally distributed. Therefore, if the p-value is less than 0.05, then the null hypothesis is rejected and it means the data tested are not from a normally distributed population.

The formula for the test: $W = \frac{(\Sigma_{i=1}^{n} a_i x_{(i)})^2}{\Sigma_{i=1}^{n}(x_i - \bar{x})^2}$ (Shapiro and Wilk, 1965)

In this formula:

$x_{1,\dots}x_n$ are data to be checked for normal distribution

$x_{(i)}$ is the smallest number in the data

$\bar{x} = (x_1 + \cdots + x_n)/n$ is the sample mean

The constants a_i are calculated from $(a_{1,\dots,}a_n) = \frac{m^T V^{-1}}{(m^T V^{-1} V^{-1} m)^{1/2}}$

Here $m = (m_{1,\dots,}m_n)^T$ are from the order statistics of independent and identically distributed random variables sampled from the standard normal distribution. V is the covariance matrix obtained from the order statistics.

Wilcoxon signed-rank test

Wilcoxon signed-rank test is a non-parametric alternative to the t-test that does not assume data are normally distributed. The null hypothesis for this test is that the two population means are equal, with difference between the pairs follows a symmetric distribution around zero. Thus if the p-value is less than 0.05, it means the null hypothesis is rejected and the alternative hypothesis (the means are different) is accepted.

The test begins by pairing the two data sets (e.g., 1 and 2) and transforming each instance of A1—A2, B1—B2,…, Z1—Z2 into its absolute value, where all the positive and negative signs are removed. In this test, zero difference between two data points (e.g., A1—A2 = 0) is eliminated from consideration, because it provides no useful information. The remaining absolute differences are then ranked from lowest to highest, with tied ranks also included in the ranking order.

The formula for or calculating the ranked data: W= $\sum_{i=1}^{Nr}[\mathrm{sgn}(x_{1,i} - x_{2,i}) \cdot R_i]$ (Gardener 2012)

In this formula:

The number of pairs $i = 1, \dots, Nr$.

The absolute value of each pair is calculated $|x_{1,i} - x_{2,i}|$ and sgn is the sign function.

R_i donates the absolute value into the ranking.

Tukey HSD test

Tukey HSD test uses the Studentized range statistic. Studentized range distribution is showing below.

$q = \frac{(\bar{x}_{max} - \bar{x}_{min})}{s \sqrt{2/n}}$ (Gardener 2012)

S expressed in the formula is the sample variance from the samples

The value q is affected by three factors:

1) the type I error rate.

2) the number of populations

3) the degrees of freedom

The Tukey HSD test uses these critical values of q to calculate how large the difference between the means of any two particular groups. The difference is presented in order to be regarded as significant.

C.6 R-scripts Used in Data Analysis

Sensory Experiment Data Analysis

```
> getwd()

[1] "/Users/yiyang/Documents/SensoryDataAnalysis"

> setwd("~/Documents/SensoryDataAnalysis/")

> BETsDay1Day2<- read.csv ("BETsDay1Day2.csv",header=T,sep=",")

> names(BETsDay1Day2)

[1] "Panelists" "BETsDay1"  "BETsDay2"

> table(BETsDay1Day2$BETsDay1) #here are the BETs values obtained from the 1st experiment

 0.35  1.04  3.12  9.37 28.15

   2     5    11    10     4

> table(BETsDay1Day2$BETsDay2) #here are the BETs values obtained from the 2nd experiment

 0.35  1.04  3.12  9.37 28.15

   2     8    11     5     6

> x<-log10(BETsDay1Day2$BETsDay1)

> y<-log10(BETsDay1Day2$BETsDay2)

#here the BET values are transformed to into the base 10 logarithm to be consistant with the
method provided ASTM E679.

> hist(x)
```

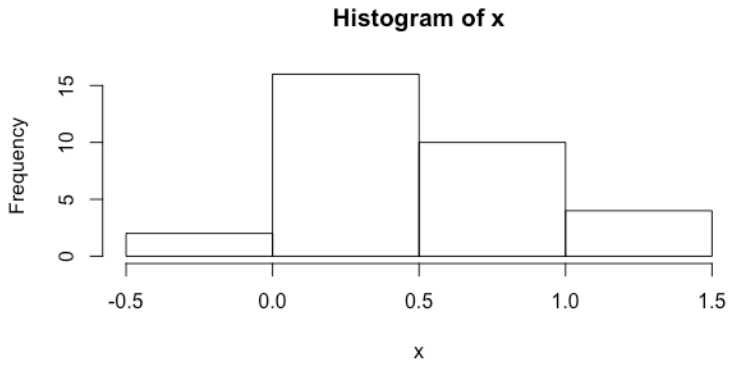

Figure C.1 Day 1, frequence distribution of the individual BET values (in $\log_{10}$) from the first sensory experiment (RStudio).

> shapiro.test(x)

Shapiro-Wilk normality test

data: x

W = 0.91531, p-value = 0.01554

#*P*-value is less than 0.05, therefore reject the null hypothesis.

> qqnorm(x)

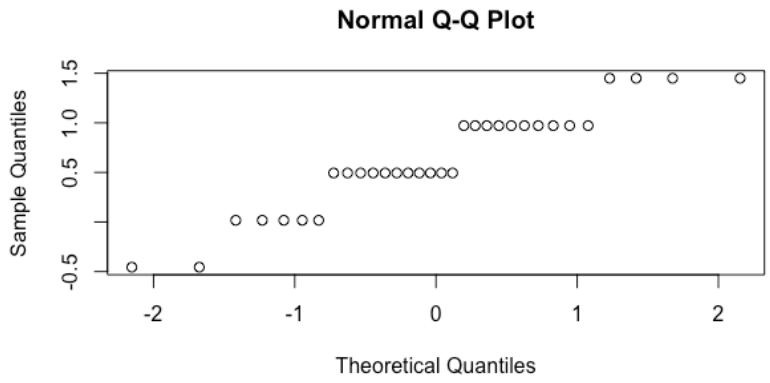

Figure C.2 Q-Q plot confirms that the BET values from the first experiment (in $\log_{10}$) are not normally distributed (RStudio).

> hist(y)

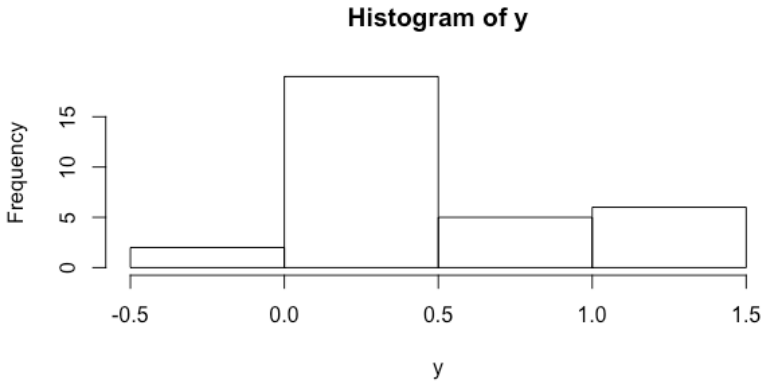

Figure C.3 Day 2, frequence distribution of the individual BET values (in $\log_{10}$) from the second sensory experiment (RStudio).

> shapiro.test(y)

Shapiro-Wilk normality test

data: y

W = 0.90277, p-value = 0.007291

#*P*-value is less than 0.05, therefore reject the null hypothesis.

>qqnorm(y)

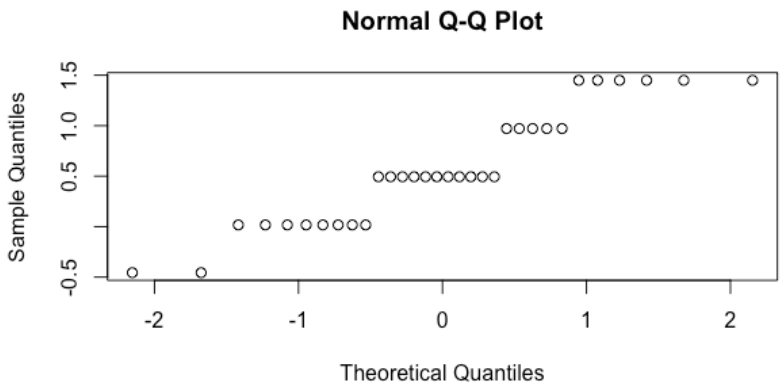

Figure C.4 Q-Q plot confirms that the BET values from the second experiment (in $\log_{10}$) are not normally distributed (RStudio).

> wilcox.test(x,y) # Wilcoxon rank sum test demonstrates *P-value* > 0.05.

Wilcoxon rank sum test with continuity correction

data: x and y

W = 553.5, p-value = 0.569

alternative hypothesis: true location shift is not equal to 0

Instrumental Experiment Data Analysis

Data From Tannin Samples

```
> getwd()
[1] "/Users/yiyang/Documents/SensoryDataAnalysis"
> setwd("~/Documents/SensoryDataAnalysis/")
> GCAnalysis<- read.csv ("GCAnalysis.csv",header=T,sep=",")
> names(GCAnalysis)
[1] "Tannin" "EH"
> table(GCAnalysis$Tannin)
 0.6  1.8 16.2  5.4    C
   2    2    2    2    2
> table(GCAnalysis$EH)
370.3 373.3 381.8 383.7 393.5 394.8 428.9 431.5 435.3 435.6
    1     1     1     1     1     1     1     1     1     1
> shapiro.test(GCAnalysis$EH)

        Shapiro-Wilk normality test

data:  GCAnalysis$EH
W = 0.84253, p-value = 0.04731  # P-value <0.05
> qqnorm(GCAnalysis$EH)
```

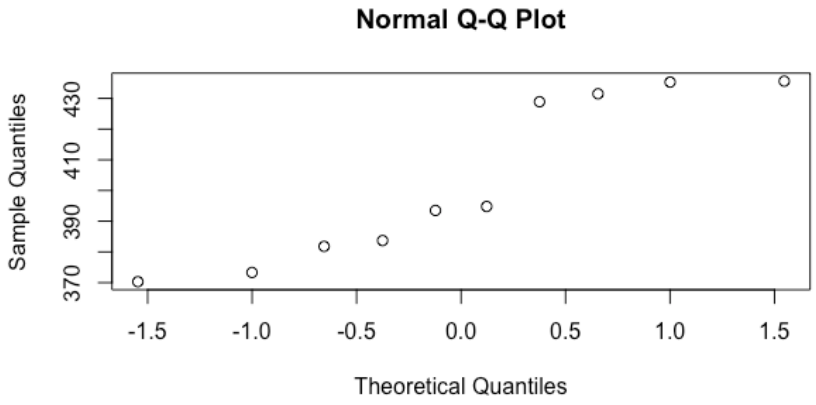

Figure C.5 Q-Q plot illustrates the distribution of the GC-MS data from the control and tannin samples(RStudio).

> Modell <- aov ((GCAnalysis$EH) ~ GCAnalysis$Tannin, data = GCAnalysis)

> summary(Modell)

```
                Df Sum Sq Mean Sq F value  Pr(>F)
GCAnalysis$Tannin  4   6509  1627.3   769.4 3.7e-07 ***
Residuals          5     11     2.1
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

> TukeyHSD(Modell)

Tukey multiple comparisons of means

95% family-wise confidence level

Fit: aov(formula = (GCAnalysis$EH) ~ GCAnalysis$Tannin, data = GCAnalysis)

$`GCAnalysis$Tannin`

Comparisons	diff	lwr	upr	p-adj
1.8-0.6	10.95	5.1160526	16.78395	0.0035036
16.2-0.6	58.40	52.5660526	64.23395	0.0000016
5.4-0.6	22.35	16.5160526	28.18395	0.0001125
C-0.6	63.65	57.8160526	69.48395	0.0000010
16.2-1.8	47.45	41.6160526	53.28395	0.0000052
5.4-1.8	11.40	5.5660526	17.23395	0.0029121
C-1.8	52.70	46.8660526	58.53395	0.0000030
5.4-16.2	-36.05	-41.8839474	-30.21605	0.0000151
C-16.2	5.25	-0.5839474	11.08395	0.0733710
C-5.4	41.30	35.4660526	47.13395	0.0000095

> par(mfrow=c(2,3))

> plot(Modell)

> qqnorm(resid(Modell))

> qqline(resid(Modell))

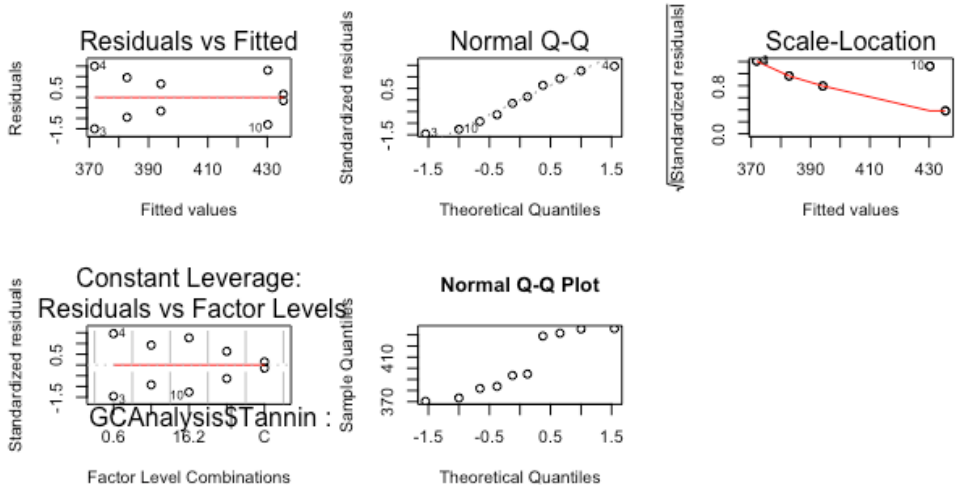

Figure C.6 Q-Q plot illustrates the distribution of the residuals of the headspace concentration data from the Tukey HSD test. In the Residuals vs Fitted plot, it can be seen that a random scatter of points with no detectable pattern. In addition, in the Normal Q-Q plot of the standardised residuals, it can be seen that the residuals are normally distributed. After checking the model assumptions and fit, it can be concluded that none of these assumptions is violated, so the results from the Tukey HSD tests can be accepted.

Data From Tannin Samples with Artificial Saliva

> getwd()

[1] "/Users/yiyang/Documents/SensoryDataAnalysis"

> setwd("~/Documents/SensoryDataAnalysis/")

> GCAnalysisTanninwithAS<- read.csv ("GCAnalysisTanninwithAS.csv",header=T,sep=",")

> names(GCAnalysisTanninwithAS)

[1] "Tannin" "EH"

> table(GCAnalysisTanninwithAS$Tannin)

0.6 1.8 16.2 5.4 C

2 2 2 2 2

> table(GCAnalysisTanninwithAS$EH)

361.9 375.9 381.4 382.8 389.1 391.8 392.8 393.1 435.8 438.8

1 1 1 1 1 1 1 1 1 1

> shapiro.test(GCAnalysisTanninwithAS$EH)

Shapiro-Wilk normality test

data: GCAnalysisTanninwithAS$EH

W = 0.84382, p-value = 0.04903

> qqnorm(GCAnalysisTanninwithAS$EH)

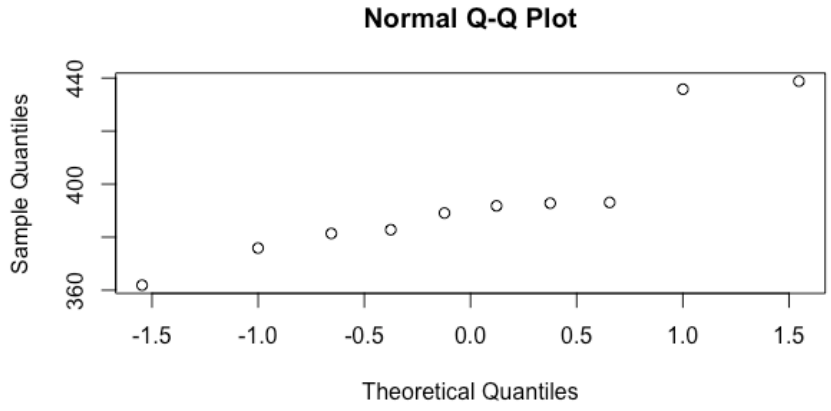

Figure C.7 Q-Q plot illustrates the distribution of the GC-MS data from the control and tannin samples added with artificial saliva (RStudio).

> Modell <- aov ((GCAnalysisTanninwithAS$EH) ~ GCAnalysisTanninwithAS$Tannin, data = GCAnalysisTanninwithAS)

> summary(Modell)

Df Sum Sq Mean Sq F value Pr(>F)

*GCAnalysisTanninwithAS$Tannin 4 5319 1329.8 62.04 0.000189 ****

Residuals 5 107 21.4

---

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

> TukeyHSD(Modell)

Tukey multiple comparisons of means

95% family-wise confidence level

Fit: aov(formula = (GCAnalysisTanninwithAS$EH) ~ GCAnalysisTanninwithAS$Tannin, data = GCAnalysisTanninwithAS)

Comparisons	diff	lwr	upr	p-adj
1.8-0.6	8.35	-10.222011	26.922011	0.4597746

16.2-0.6	10.85	-7.722011	29.422011	0.2679080
5.4-0.6	-13.20	-31.772011	5.372011	0.1579641
C-0.6	55.20	36.627989	73.772011	0.0004010
16.2-1.8	2.50	-16.072011	21.072011	0.9786429
5.4-1.8	-21.55	-40.122011	-2.977989	0.0280746
C-1.8	46.85	28.277989	65.422011	0.0008801
5.4-16.2	-24.05	-42.622011	-5.477989	0.0179094
C-16.2	44.35	25.777989	62.922011	0.0011414
C-5.4	68.40	49.827989	86.972011	0.0001380

> par(mfrow=c(2,3))

> plot(Modell)

> qqnorm(resid(Modell))

> qqline(resid(Modell))

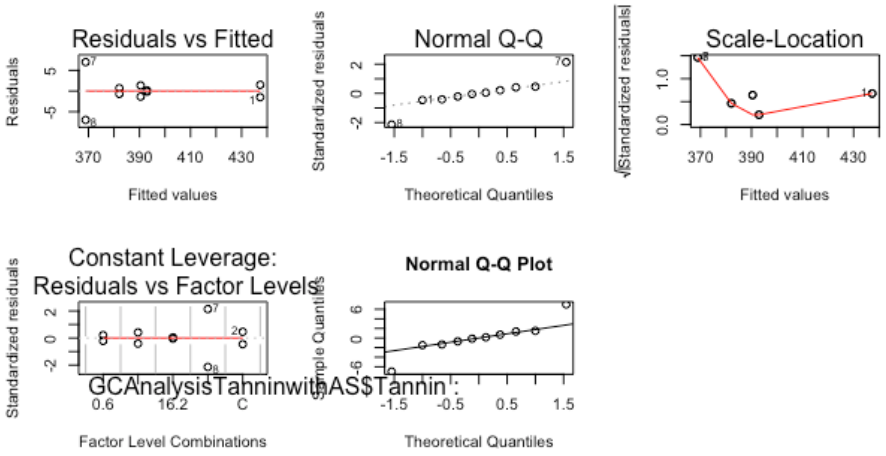

Figure C.8 Q-Q plot illustrates the distribution of the residuals of the headspace concentration data (under the influence of artificial saliva) from the Tukey HSD test. In the Residuals vs Fitted plot, it can be seen that a random scatter of points with no detectable pattern. In addition, in the Normal Q-Q plot of the standardised residuals, it can be seen that the residuals are normally distributed. After checking the model assumptions and fit, it can be concluded that none of these assumptions is violated, so the results from the Tukey HSD tests can be accepted.

Comparison of the Headspace Concentrations Before and After the Addition of Artificial Saliva

```
> getwd()
[1] "/Users/yiyang/Documents/SensoryDataAnalysis"
> setwd("~/Documents/SensoryDataAnalysis/")
> EHGC<- read.csv ("EHGC.csv",header=T,sep=",")
> names(EHGC)
[1] "Tannin"       "EHDifference"
> table(EHGC$Tannin)
 0.6  1.8  5.4 16.2
   2    2    2    2
> table(EHGC$EHDifference)
 6.3   10 10.3 11.4 19.6 32.7 36.6 37.3
   1    1    1    1    1    1    1    1
> shapiro.test(EHGC$EHDifference)

        Shapiro-Wilk normality test

data:  EHGC$EHDifference
W = 0.83692, p-value = 0.07001
> qqnorm(EHGC$EHDifference)
> Modell <- aov ((EHGC$EHDifference) ~ EHGC$Tannin, data = EHGC)
> summary(Modell)
             Df Sum Sq Mean Sq F value  Pr(>F)
EHGC$Tannin   1  952.7   952.7   24.11 0.00268 **
Residuals     6  237.1    39.5
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

> TukeyHSD(Modell)

Tukey multiple comparisons of means

95% family-wise confidence level

Fit: aov(formula = (EHGC$EHDifference) ~ EHGC$Tannin, data = EHGC)

$`EHGC$Tannin`

	diff	*lwr*	*upr*	*p adj*
T1.8-T0.6	*-2.7*	*-22.381647*	*16.981647*	*0.9394428*
T16.2-T0.6	*26.1*	*6.418353*	*45.781647*	*0.0193094*
T5.4-T0.6	*15.3*	*-4.381647*	*34.981647*	*0.1072319*
T16.2-T1.8	*28.8*	*9.118353*	*48.481647*	*0.0136110*
T5.4-T1.8	*18.0*	*-1.681647*	*37.681647*	*0.0662443*
T5.4-T16.2	*-10.8*	*-30.481647*	*8.881647*	*0.2568413*

> par(mfrow=c(2,3))

> plot(Modell)

> qqnorm(resid(Modell))

> qqline(resid(Modell))

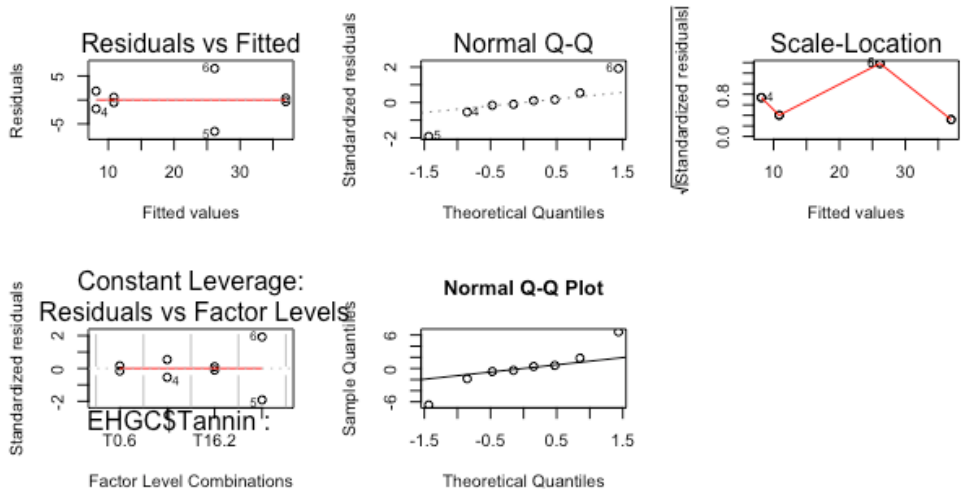

Figure C.9 Q-Q plot illustrates the distribution of the residuals of the comparisons of the headspace concentration data (before and after the addition of artificial saliva) from the Tukey HSD test. In the Residuals vs Fitted plot, it can be seen that a random scatter of points with no detectable pattern. In addition, in the Normal Q-Q plot of the standardised residuals, it can be seen that the residuals are normally distributed. After checking the model assumptions and fit, it can be concluded that none of these assumptions is violated, so the results from the Tukey HSD tests can be accepted.

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